ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES
FISCAL YEAR 1973

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service National Institutes of Health

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ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR

National Institute of Arthritis, Metabolism, and Digestive Diseases

Fiscal Year 1973

by

Dr. J. E. Rall

For the National Institute of Arthritis, Metabolism, and Digestive Diseases, 1972-73 has been a year of dislocation, of work and achievement, and of recognition. Dislocations have arisen because of constrictions on positions so that we have been forced to reduce the number of scientists in the Institute. In addition, restrictions have been placed on promotions so that hard decisions have had to be made about the very limited number of promotions which have been possible for the staff of this Institute.

Slower and more subtle has been the intellectual dislocation, or rethinking of biomedical research which most scientists have undergone in the last few years as a result, primarily, of the increasing pressure of a public which pays the cost of biomedical research and which desires relevancy and results for the investment of their monies. It is appreciated no less by the scientists that they owe an obligation to the community which supports their work to produce results which have some relationship to the problems that the community faces and that their work may ameliorate. In addition, it has also become apparent that as the federal budget for biomedical research has increased to several billion dollars, the disposition in more specific terms of that budget has begun to involve political considerations. It is perhaps not as well appreciated that policical considerations, in spite of the pejorative aspects of the adjective, are in fact an inextricable part of a democratic society.

The result of all these tendencies has been, for scientists, biologists, physicians, to examine the specifics of their work in relationship to societal needs. In many instances it is quite apparent that it is not profitable to attempt to change fields. It is not sensible for an x-ray crystallographer to attempt to diagnose arthritis; it is not reasonable for a synthetic organic chemist to take care of patients with diabetes. It is, however, a healthy sign when a basic scientist takes both a hard and a comprehensive look at the relationship of his work to biomedical research in the continuum from the most fundamental physicalchemical laws to derangements of function producing what we know as human disease.

The results of these reflections, these revaluations, and redirections of research going on fairly generally not only in our Institute but throughout the country, will probably not soon be seen. Nonetheless they portend changes which will probably be seen as practical results in disease control in the years ahead.

Meanwhile, work in highly specific areas, as can be seen from the following summaries, has been extensive, productive and important. Some of this work has been recognized by awards in a variety of fields. To be mentioned are the Philip Hench Award given to Dr. John L. Decker for his work in the field of arthritis; the Martin Luther King, Jr. Award given to Dr. M. Murayama for his work on sickle cell anemia; the Jacobeus Prize awarded Dr. Martin Rodbell for his work on hormone action; the Stouffer Prize awarded Dr. Robert S. Gordon, Jr. for his work on lipid metabolism; and the Nobel Prize given Dr. Christian B. Anfinsen for his work on protein structure.

ANNUAL REPORT SUMMARY

LABORATORY OF NUTRITION AND ENDOCRINOLOGY, 1973

The laboratory office, the Office of the Chief and the Developmental Biochemistry and Vitamin Metabolism Sections have moved to new quarters in the basement floor of building 6. Only the major elements of the Pilot Plant operation remain in Building 3. It is expected(?) that the Pilot Plant will also be moved to its new quarters in building 6 this next fiscal year.

PILOT PLANT UNIT

Large-Scale Processing of Biological Materials.

During the past year, 252 requests by NIH investigators were processed. 221 kilogram quantities (54,600 liters of cultures) of 16 microorganisms were produced. The microorganisms grown during this period included bacteria and yeast and consisted of wild type and mutant strains of Aerobacter aerogenes, Bacillus amyloliquefaciens, Bacillus subtilis, Citrobacter sp., Corynebacterium acnes, Comamonas sp., Erwinia cartovora, Escherichia coli, Haemophilus influenzae, Pediococcus cerevisiae, Pseudomonas sp., Saccharomyces cerevisiae, Salmonella typhimurium, Streptococcus lactis and Streptococcus mutans. Some of the organisms were used as hosts for bacteriophage production, such as fd, MS2, 80, lambda, R17 and T4. Others, such as Bacillus amyloliquefaciens, were used to produce extracellular enzymes. Microorganisms were grown 86 times in the 300-liter, 38 times in the 10-liter, 24 times in the 1100-liter and 41 times in the 50-liter fermentors.

Studies were continued with the NICHD for polysaccharide production by Haemophilus influenzae and cross-reacting organisms such as Escherichia coli. A polysaccharide fraction containing the protective antigen of typhoid fever was isolated from a non-pathogenic organism, Citrobacter sp. E. coli W6 spheroplasts were produced for the NHLI three times, using 10-, 50-, and 300-liter fermentors for each preparation. The Gaulin laboratory homogenizer was used 47 times to rupture suspensions of yeast or bacterial cells. Large-scale processing activities consisted of the preparation of Histamine-N-Methyltransferase enzyme from 1.8 kg guinea pig brains (repeated with 2.6 Kg), the extraction of hormones from parathyroid substance in six, one kilogram preparations, the isolation of an inhibitor, barstar, from 15 kg Bacillus amyloliquefaciens, the preparation (twice) of Deoxyribonucleotide polymerizing enzyme from 10 lbs calf thymus glands, the isolation and chromatographic purification of Histidase from 2 kg of a pseudomonad ATCC 11299b, the processing of 5.8 kg Koelreutria paniculata seeds for an alcohol-soluble substance and the isolation of rennin from 20 lbs of hog kidney. The KII Zonal centrifuge was used to band cell membranes from bovine thyroid glands and for the separation of E. coli J5 membranes. Efforts were made to separate and purify red blood cell "ghosts". The large capacity and turbine Sharples centrifuges were used to separate cells from suspensions, such as Basidiomycetes, Lactobacillus fructosus and red blood cell "ghosts" from 30 units of hemolyzed human red blood cells. Various quantities of plant and and animal tissues such as bovine tendon and calf muscle were ground in the assorted laboratory mills.

GERM-FREE RESEARCH UNIT

Studies in Experimental Nutrition

Cycasin, which is not toxic to adult germfree rats, is toxic to conventional rats, to very young germfree rats and to ex-germfree rats infected with bacteria capable of converting the compound to its aglycone. Germfree rats, infected with bacteria unable to make this conversion, react like germfree rats and show no toxicity to cycasin. The aglycone of cycasin, however, is toxic to germfree as well as conventional rats at any age. Although adult rats are unable to convert cycasin to aglycone without bacteria, the very young animal has this capability which is lost early in life. Current results indicate that between sixteen and twenty-five days of age, the ability of the germfree rat to convert cycasin to the toxic aglycone is sharply reduced, although some tumors were observed in animals receiving cycasin at 35 days. (G. Laqueur and E. G. McDaniel).

Studies on the interrelationship between vitamin A and zinc were initiated because of the similarities of symptoms in deficiency states. Bacteria have a marked influence upon the development and severity of deficiency symptoms, survival time and, perhaps, upon the actual requirement of both vitamin A and zinc. In both cases, as progressively lower levels of these factors are attained, germfree and conventional animals respond more alike, suggesting that the differences result from more efficient utilization by germfree animals or from competition by bacteria for nutritional factors. At borderline levels of zinc, conventional or ex-germfree rats required a higher level of the mineral to equal the growth of germfree rats.

Doubly-deficient animals, supplemented with zinc alone, after a short period of rapid growth, show a very early and acute vitamin A deficiency, more severe than that attainable by vitamin A depletion alone. This early period of rapid growth may actually serve to deplete the vitamin A stores. Severe A deficiency is not completely reversible whether accomplished with the aid of zinc deficiency or not. Tissues of these animals are being examined histologically.

Additional studies of doubly-deficient rats suggest that zinc is required for the mobilization and transport of vitamin A from the liver to blood and, perhaps, to specific sites of activity in other tissues. Zinc or zinc plus vitamin A, but not A alone, stimulate growth in doubly-deficient animals. However, growth restriction by limiting food intake during zinc supplementation appears to decrease the ability to mobilize vitamin A, suggesting that new tissue formation is required to overcome the damage induced by the deficiency states. (J. Smith, VA Hospital, and E. G. McDaniel).

SECTION ON DEVELOPMENTAL BIOCHEMISTRY

Structure and Function of Nucleic Acids

In attempts to increase the capacity of gel electrophoresis (GE), positive or negative substituents were incorporated into the polyacrylamide matrix, resulting in gels characterized by "creeping", i.e., disruption of gels and movement out of tube upon prolonged electrophoresis. Smaller amounts of substituents incorporated decreased creeping but also lowered the capacity for additional oligonucleotides, to a point close to that of unsubstituted gels. Among "balanced" gels, combinations of acrylic acid and polyethyleneimine gave the best results with respect to improved resolution (increase in total number of oligomer bands over that obtained with unsubstituted gels). However, while resolution was improved, capacity, the primary objective, was not.

fd DNA from light fd particles moves like fd DNA from mature virus upon sucrose gradient or CsCl sedimentation. Partial hydrolysates with micrococcal nuclease and GE at various gel concentrations (6-18%) shows similar patterns of oligonucleotides. Differences between the DNAs, if any were not detected.

While both intact MS2 and fd virus are resistant to RNases or DNases, respectively, modification of the protein coat with SDS, succinic anhydride, N-bromosuccinimide (NBS), or Clelands reagents (dithioerythritol or dithiothreitol) provides viral derivatives that allow enzymatic hydrolysis of the nucleic acid within the modified viral particle. With MS2, RNase T1 and Barnase gave good patterns of large oligonucleotides by GE at 6 and 12% gel concentration. Unexpectedly, the same viral derivatives of MS2 provided no protection against RNase T_2 or pancreatic RNase hydrolysis. On the other hand, micrococcal nuclease was not able to attack the modified MS2 at all. With RNase T_1, oligomer fractions of the size range of tRNA were obtained in 51-86% yields, better than the yields obtained with MS2 RNA itself, where the best yields did not exceed 28% of the total RNA. The larger oligonucleotides remaining could not be recovered from DEAE-cellulose used to fractionate the smaller oligomers. More stringent and different conditions for the derivatization of fd virus were required. SDS treatment was not used since micrococcal nuclease (MN), the enzyme of interest, was inactive in the presence of SDS. Succinic anhydride in aqueous buffers had no effect on fd, as measured by sucrose gradient sedimentation of DNA of MN-treated fd derivatives. NBS first produced aggregation (increase in S_{20}) and then monomer formation (decrease in S_{20} over native fd) with increasing amounts of reagent to virus. However, MN hydrolysis gave no distinct oligomer patterns on GE, only smears, a result, perhaps of the decreased solubility of fd at pH 3-4, the required pH for NBS reaction with tryptophan or tyrosine, even in 7 M urea. Oxidation of exposed Ap residues in DNA may also occur, even though this reaction is usually carried out at higher temperature (boiling for 1-2 hours). Work is in progress on the modification of fd in 7 M urea with succinic anhydride, as well as with DTE or DTT. (G. W. Rushizky and H. A. Sober).

Protein-Protein Interaction: Structure and Function of an Extracellular Ribonuclease (Barnase) and its Intracellular Inhibitor (Barstar).

Collaboration, begun during the tenure of a Wellcome Fellowship (on LWOP) at the Sub-division of Protein Chemistry, Laboratory of Molecular Biology, Medical Research Council, Cambridge, England, has been continued and has, by X-ray crystallography, resulted in a 4.5 Å solution of the barnase structure by studies of a single crystal, using the special three-fold symmetry of the barnase crystal to obtain phase information. It has not been possible to trace the peptide chain at this resolution, nor does it appear that further resolution will be possible with this technique. However, two good isomorphous replacements have been obtained, using gold cyanide and mercury salycilate. Full data at

2.5 Å resolution has been taken for these two as well as for the "native" crystal and a solution at that resolution is anticipated. (D. M. Blow (M.R.C.) and R. W. Hartley).

A peptide complementation system for barnase has been discovered. Barnase (1-103), missing eight residues from its C-terminal, binds the peptide (88-110) with an assocation constant in the order of 10^{-5} and 10^{-6} and with restoration of at least 50% of its ribonuclease activity. The peptide (99-108) also complements, but requires a thousand-fold excess. No complementation is obtained with the missing octapeptide (103-110) itself. (R. C. Sheppard (M.R.C.) and R. W. Hartley).

The reaction between barnase and the detergent sodium dodecyl sulfate can be represented by a simple model involving a reversible, all-or-none conformational transition from which the number of detergent molecules bound to each form can be determined. A reversible transition produced by the cationic detergent, cetyltrimethylammonium bromide, appears to be somewhat more complex and produces entirely different spectral changes.

Changes in the bacterial growth medium, introduced for engineering reasons, have allowed the production of a substantial quantity (20-30 mg) of barnase-A. This is an active, but chromatographically different, form of barnase which is probably a precursor of the barnase normally isolated. (R. W. Hartley).

Protein-Nucleic Acid Interaction: Chromatin Structure and Restriction in Eukaryotic Cells

The mode of interaction of the five main histones with DNA in native chromatin was studied after chromatin acetylation by isolation of the modified histones and determination of the amount and localization of the incorporated acetate after peptide mapping. Without prior isolation of the modified histones, ambiguities in compositional analysis and in isolation of unique peptide sequences occurred, making the determination of modified lysyl residues uncertain. Conventional histone fractionation methods, such as chromatography on cation exchange celluloses and resins, gel filtration and differential solvent extraction, have proven to be inapplicable to the fractionation of acetylated proteins. Five main histone fractions can be resolved, however, by hydroxylapatite chromatography of the sodium dodecyl sulfate complexes of the modified proteins. The separated peaks differ in the amount of acetate present per unit mass of protein and appear to be nearly homogeneous by electrophoretic analysis. Sufficient quantities of these purified, modified proteins are now being accumulated to allow localization of nonbound lysyl residues of individual histones in chromatin.

The use of nuclease digestion as a tool for the study of chromatin structure was explored using three different deoxyribonucleases, DNase I, DNase II and micrococcal nuclease. The amount of DNA digested differed for each of the three enzymes and was a function of other experimental conditions, including the enzyme substrate ratio, ionic strength and amount of divalent cation present. The structure of chromatin can not be rigidly defined in terms of amount of "accessible" DNA by such experiments, but that factors such as the mode of enzyme action and the strength of protein-nucleic acid interactions, both, in part, a function of ionic conditions, must be considered. (R. T. Simpson, R. Seale and L. Propst).

Fractionation of Chromatin

Chromatin, subjected to ECTHAM-cellulose chromatography (Reeck et al., 1972) can be separated into early and late-eluted fractions which have been shown to be more condensed and more extended, respectively, than unfractionated chromatin. Despite these striking differences in physical properties between early and late eluted fractions, differences in protein content, while present, are rather subtle. Condensed and extended chromatin fragments contain comparable amounts of all histones, except for histone F1. Only approximately 25% as much of this histone occurs in the extended chromatin as appears in the condensed fractions of each chromatin. This, in large part, accounts for the lower histone/DNA mass ratio of 0.8 in the extended chromatin fractions as compared to the ratio of 1.1 to 1.2 in the condensed fractions.

Extended and condensed calf thymus chromatin fractions contain comparable amounts of nonhistone proteins (0.2 g/g DNA), but extended rabbit liver chromatin has considerable more nonhistone than the condensed fraction (2.5 g/g DNA compared to 1.0 g/g DNA). In addition to these quantitative differences, in rabbit liver chromatin, there are clear-cut qualitative differences between the nonhistones of condensed and extended fractions. The most striking is the presence of a particular, rather high molecular weight nonhistone protein occurring exclusively within the extended fraction. Differences, which appear to be species specific, are also observed between the nonhistones of extended and condensed fractions of rabbit kidney and HeLa chromatins.

Because of the postulated association between histone acetylation and phosphorylation and gene activation, and because of the possible enrichment of transcribed DNA sequences in extended chromatin, the histones of condensed and extended chromatin fractions of rabbit liver and calf thymus were examined to determine the distribution of acetylated and phosphorylated histone molecules. In each chromatin, the extended and condensed fractions showed identical amounts of the modified histones in comparison to the parent molecules.

The prominent appearance of a particular nonhistone protein in the extended fraction of rabbit liver chromatin has prompted the isolation of this protein. A remarkable purification can be obtained by extracting chromatin with 1.5 M NaCl, removing most of the histones from DNA. Of the nonhistones, however, the protein of interest is almost exclusively extracted. Dialysis of these extracted proteins against 0.01 M Tris·Cl, pH 8.0, precipitates the histones and the nonhistone is left in solution. The nonhistone can be further purified to a state of apparent homogeneity by chromatography on DEAE-cellulose. Thus, under relatively mild conditions,and in a relatively simple procedure, a nonhistone protein, exclusively associated with the extended fraction of rabbit liver chromatin, has been isolated. (G. Reeck, R. T. Simpson, and H. A. Sober).

Protein-Nucleic Interaction: Chromatin Structure and Restriction in Eukaryotic Cells.

The structure and function of chromatin fractions obtained by chromatography on ECTHAM-cellulose of sonicated chromatin has been studied to examine the functional capacities for both in vivo and in vitro DNA-dependent RNA synthesis.

Determination of the in vitro "template" activity of the isolated fractions for either calf thymus nucleoplasmic or E. coli RNA polymerase under a variety of experimental conditions has led to low and variable activities with no significant differences between the various fractions. The low activity (relative to protein-free DNA), probably reflecting the small size of the chromatin pieces required for chromatographic separation, may preclude accurate assessment of their functional capacity in vitro. Significant differences have been detected in their capacity for support of in vivo RNA synthesis. When rats are labeled for ten minutes with tritiated orotic acid and the liver chromatin isolated, sonicated, and centrifuged to remove dissociated RNA and then chromatographed on ECTHAM-cellulose, the late-eluted, low-melting chromatin fractions contained twenty-five times the nascent RNA (relative to DNA content) of the early-eluted, high-melting chromatin segments. Supporting results have been obtained for different times of labeling and with different tissues (e.g., HeLa cells). This observation, together with those of McConaughy and McCarthy, demonstrate that the late-eluted chromatin is preferentially transcribed in vivo, while the early-eluted chromatin segments are relatively repressed.

Examination of the solution conformation of chromatin segments which are transcribed (late-eluted) and repressed (early-eluted) have shown that the circular dichroism spectra of rabbit liver and calf thymus chromatin fractions differ from those of both DNA and unfractionated chromatin. In both cases, the early-eluted chromatin has an even lower ellipticity at 280 nm than unfractionated chromatin while the last-eluted material has an ellipticity much nearer that of protein-free DNA. This decreased ellipticity of chromatin has been attributed to assumption of the C form or to supercoiling. Whatever the mechanism, repressed chromatin has a conformation different from that of isolated DNA while transcribed chromatin has a structure far more like that of the protein-free nucleic acid. Confirmatory results have been obtained by other physicochemical methods of structure evaluation.Light scattering studies indicate similar particle weights for the two chromatin fractions but that repressed chromatin exists in a more compact shape than transcribed chromatin. Similarly, sedimentation of chromatin fractions in either linear 5-50% sucrose gradients or self-generating D_2O gradients leads to differing properties for early- and late-eluted chromatin segments, with repressed chromatin sedimenting more rapidly than unfractionated chromatin, (21 S vs 18 S in D_2O) and transcribed chromatin, sedimenting more slowly (9S). Since the segments are approximately of the same size, such differences in sedimentation must arise from differences in particle shape, the repressed chromatin being more compact in structure with the transcribed sequences in a more extended conformation.

The current investigations indicate that ECTHAM-cellulose chromatography separates segments of chromatin which differ in both functional and physicochemical properties. Early-eluted chromatin is repressed in vivo, and exists in a compact, possibly supercoiled, structure. In contrast, late-eluted chromatin segments are preferentially transcribed in vivo and show a solution conformation more nearly that of isolated DNA. The experimental results are consistent with models for eukaryotic gene regulation in which supercoiling of DNA sequences is part of the mechanism of repression of transcription. (R. T. Simpson, I. Polacow, G. Reeck and L. Propst).

Metabolic Studies on Chromatin: Synthesis Assembly and Turnover.

In the presence of sufficient cycloheximide (CH) to block protein synthesis

DNA synthesis in HeLa cells proceeds for about one hour at a linear rate, 20% that of control. Identical results with puromycin indicate that the decreased rate is more likely due to a lesion in protein synthesis than to interference with replication by cycloheximide. DNA synthesized during CH blockage of protein synthesis was demonstrated by several criteria to be a continuation of the normal replication process and not a repair phenomenon. CH·DNA synthesis is abolished by hydroxyurea which has been reported not to affect repair replication. Radioactivity, incorporated either before or after administration of CH, sediments with the bulk of DNA and not with smaller fragments in alkaline sucrose gradients. Cells, pulsed with BUdR and then treated with cycloheximide and isotopic thymidine, contain labeled DNA which cosediments preferentially with heavy DNA, indicating covalent linkage to recently replicated DNA.

DNA synthesized in the absence of protein synthesis should be free of chromatin proteins and thus more susceptible to nuclease attack than normal chromatin DNA. Indeed, by pancreatic DNase I digestion, CH·DNA was more susceptible to enzymatic hydrolysis than controls, but less so than free DNA. This intermediate value of "digestibility" implied that non-bound chromatin protein pools were present. Cells were pulse-labeled with H^3-leucine for 5 minutes and chased by addition of CH. In the ensuing 60 minutes, both histone and nonhistone chromatin proteins increased in specific activity by 30%. Polyacrylamide gels sliced for radioactivity assay revealed no preferential incorporation of specific histones or non-histone proteins.

Current efforts are also directed toward isolation and characterization of histone messenger RNA, notably histone F1 mRNA. Histone F1 is chemically distinct from other histones by virtue of its high lysine content: it has a molecular weight approximately twice that of other histones and is markedly more antigenic. Coupled with the size differentiation of histone messengers from the general RNA population, their appearance at a discrete interval in the cell cycle, their sensitivity to inhibitors of DNA synthesis, and the reported lack of 3'-terminal polyadenylates, histone messengers provide a vehicle for study of control of histone synthesis, especially with regard to DNA synthesis. Initial efforts are directed toward assessment of messenger RNA. (R. Seale).

SECTION ON ENDOCRINOLOGY

Hormonal Induction of Diabetes with Exogenous Hormones.

Most studies have been concerned with changes in levels of blood glucose and serum and pancreatic insulin in rats injected with diabetogenic hormones. Some of the rats also received 0.5% tolbutamide in the diet. When possible, dosages were large enough to produce glucosuria in 3-5 days. The principal effect of tolbutamide was to lower pancreatic insulin level. The main action of bovine growth hormone (BGH) was to increase serum insulin by 24 hours without altering blood glucose. Pancreatic insulin concentration gradually increased about 50% by 5 days.

Cortisol and dexamethasone caused a rapid 3-4 fold rise in serum insulin level during the first 24 hour period and maintained this high level, usually, for 5 days. In spite of the high serum insulin level, there was little change in blood sugar during the first 24 hours. During subsequent days, there was a rise in blood sugar to over 200 mg/100 ml with glucosuria by day 3-4. Pancreatic insulin changed little during the first two days but fell thereafter to about 50% of normal. Synergistic combinations of BGH with cortisol or dexamethasone produced similar but larger changes than did the individual adrenal hormones. These changes were further magnified by tolbutamide. Without exception, there was a uniform return of the pancreatic insulin concentration toward normal by day 5 with hormonal treatment.

These data demonstrate the great capacity of the rat to maintain the insulin concentration in the pancreas and, especially, the ability of β cells to produce additional insulin in response to persistent hormonal treatment. Tolbutamide reduced by a factor of about 2 the dosage necessary to induce glucosuria in all hormonal treatments. It also reduced the time of appearance of glucosuria from about 4 to 2 days and decreased by 50-75% the concentration of insulin in the pancreas at day 5. These three effects of tolbutamide are all in the direction of diabetogenesis.

Similar studies in rats with transplantable hormone-producing pituitary tumors have shown that these chronically, endogenously stimulated rats have normal blood glucose levels (and no glucosuria) with insulin levels 4-5 times normal in serum and 3-4 times normal in pancreas, showing again the ability of rat β cells to produce insulin, perhaps, as a direct result of hormonal stimuli.

These results show that at first, diabetogenic hormones increase plasma insulin levels with little change in blood sugar or pancreatic insulin levels. With continued treatment, the pancreatic insulin levels fall (especially with tolbutamide added), and hyperglycemia and glucosuria develop in the presence of the elevated serum insulin levels. This suggests that the hormones have an initial stimulatory effect on pancreatic insulin release followed by more slowly developing systemic actions resulting in hyperglycemia. (R. W. Bates).

Lingual Lipase

Earlier studies in this laboratory showed that rat lingual serous glands secrete a very potent lipase which hydrolyzes dietary triglyceride to mostly diglyceride and FFA at pH 4.5 - 5.5 and that this enzyme accounts for most of the stomach lipolytic activity. A similar activity has been found, but at a much lower level, in posterior pharyngeal tissues, the soft palate, the dorsum of the tongue and the lateral oral pharyngeal glands. Lipolytic activity in lingual serous glands is present in the rat 1 day before birth, decreasing sharply several hours after birth and then increasing 30-fold during the next 2 weeks. The lingual lipolytic activity in adult rats is decreased 70% within 2 hours by injection of isopropyl arterenol hydrochloride and is unaffected by either bilateral resection of the glossopharyngeal nerve or bilateral sympathectomy. A similar lipolytic activity has been found in the stomach. (M. Hamosh, H. Klaeveman (A-IHD) and R. O. Scow).

Lipoprotein lipase activity and uptake of blood triglyceride by red and white skeletal muscle

Studies in several tissues (adipose, mammary, heart and diaphragm muscle) have shown that uptake of blood triglyceride is related to the level of tissue lipoprotein lipase (LPL) activity. Since skeletal muscle also utilizes circulating triglyceride for energy, the relationship between LPL activity and triglyceride uptake were examined in red and white muscle of the rat. The effects of fasting were also investigated, since fasting increases LPL activity and triglyceride uptake in heart and diaphragm muscle, and decreases both in adipose tissue.

It was found that lipoprotein lipase activity is higher in red skeletal muscle (soleus, medial head of triceps brachii and posterior belly of digastric) than in white muscle (abdominal, pectoralis, psoas major, tibialis anterior, and long head of triceps brachii), and that 1-2 days fasting increased the activity in red but not in white muscle. The level of LPL activity was closely related to uptake of blood triglyceride by the muscles in vivo, providing evidence that the enzyme is involved in triglyceride uptake in skeletal muscle. The higher uptake by red muscle, especially during fasting, is undoubtedly related to its greater dependence on fatty acids for energy. (C. Linder, S. S. Chernick and R. O. Scow).

Metabolism of chylomicron-triglyceride in perfused rat mammary gland

Studies reported last year showed that chylomicron-triglyceride is taken up and hydrolyzed by perfused lactating rat mammary gland, and that uptake and hydrolysis were both decreased 80% when the rats were unsuckled for 18 hours prior to experiment. Other studies had shown that lipoprotein lipase activity in mammary gland is greatly increased by lactation, and decreased by nonsuckling. Additional evidence that the enzyme is involved in uptake of blood triglyceride was obtained in perfused mammary tissue of hypophysectomized lactating rats; uptake and hydrolysis of triglyceride as well as lipoprotein lipase activity were negligible. Perfused mammary glands of lactating rats also hydrolyzed triglyceride in an artificial emulsion (Intralipid) but only if the emulsion had been pre-activated by incubation with rat serum. Activation prior to perfusion was necessary since the perfusing fluid was serum-free and, thus, lacked the lipoprotein needed to make emulsion triglyceride accessible to the lipolytic action of the enzyme.

Since studies with doubly-labeled, chylomicron triglyceride (^{14}C-palmitate and ^{3}H-glycerol) showed that glyceride-glycerol was retained by the tissue, it was determmined whether glycerol could be utilized by perfused mammary gland. It was found that 18% of glycerol infused at a blood concentration of 0.6 mM was incorporated into triglyceride, indicating the presence of an active glycerokinase in the mammary gland of lactating rats. The high molar ratio of FFA to glycerol, 5, found in venous blood when chylomicrons were infused, indicates that partial glycerides are formed during the initial step of triglyceride uptake. Further studies are necessary to determine if partial glycerides can be utilized by mammary alveolar cells without additional hydrolysis. (C. R. Mendelson, O. Zinder and R. O. Scow)

Effects of Lipoprotein Lipase on the Structure of Chylomicrons

Chylomicrons are lipid particles in blood which transport fatty acids as triglyceride from intestines to other tissues in the body. They consist of a triglyceride core surrounded by a thin layer of phospholipid, cholesterol, and protein. When fatty acids are taken up by tissues, chylomicron triglyceride is hydrolyzed to FFA and glycerol by lipoprotein lipase in the capillary endothelium. The rest of the chylomicron, i.e., the surface film and residual glyceride, is probably removed from the blood stream by liver.

In studies with the electron microscope in chylomicrons incubated with lipoprotein lipase from post-heparin plasma, it was found that chylomicrons assumed many different shapes as they were depleted of triglyceride by the lipolytic action of the enzyme, and that total removal of the triglyceride resulted in empty sacks of surface film. Although most of the FFA formed were immediately released to the incubation medium, some were retained within chylomicrons when FFA-binding sites on albumin were not available, suggesting a specific role for albumin in the transfer of FFA across the surface film. The surface film was visualized in sections of OsO_4-fixed chylomicrons as a thin electron-opaque line, 25-30 Å wide, in areas where the underlying triglyceride core had been replaced by zones of decreased electron opacity, and in folds of surface film extending outward from partially lipolyzed chylomicrons. Collapsed chylomicrons, depleted of core-lipid, were also seen in venous blood of lactating rat mammary gland perfused 2-3 minutes with chylomicrons. These particles, produced by the action of lipoprotein lipase within the mammary capillary bed, are undoubtedly similar to the chylomicron "remnants" and "skeletons" proposed by others (Redgrave; Berman *et al.*). The findings demonstrate that chylomicrons consist of a core of liquid triglyceride enveloped by a pliable and durable monolayered surface film, and that lipoprotein lipase reduces the triglyceride core without disrupting the surface film. (E. J. Blanchette-Mackie and R. O. Scow)

SECTION ON MEMBRANE REGULATION

Role of Guanyl Nucleotides in Activity and Hormonal Regulation of Adenylate Cyclase Systems

The adenylate cyclase system plays a key role in the actions of a variety of peptide hormones, catecholamines, and prostaglandins. The enzyme catalyzes the conversion of ATP to cyclic AMP, the so-called second messenger of hormone action. The glucagon-sensitive enzyme system in hepatic plasma membranes consists of two distinct protein components, receptor and enzyme, that are linked or coupled such that binding of glucagon to the receptor and of GTP to a regulatory site result in enhanced reactivity of ATP with the catalytic site. The nucleotide and glucagon appear to exert their actions at a common site, possibly the boundary interface between receptor and enzyme, which requires acidic phospholipids (phosphatidylserine or phosphatidylinositol) for the concerted actions of these agents. Binding of glucagon to the receptor is a rate-determining step in the binding of the nucleotide. Interaction of the two ligands at this site results in rapid release of glucagon and, in ping-pong fashion, the nucleotide. Release of the hormone and GTP results in immediate decay of enzyme activity unless continued supply of the ligands is maintained. Full receptor occupancy is required for maximal activity at concentrations of GTP below 10^{-6} M; half-maximal activation rates and receptor occupancy are obtained under these conditions with 2 to 5 nM glucagon.

In contrast, minimal receptor occupancy is required for maximal activity with 10^{-6} M GTP: half-maximal activation rates under these conditions is obtained with about 0.2 nM glucagon and less than 5% (0.2 pmoles bound hormone/mg membrane protein) receptor occupancy. The latter conditions are consistent with a rapidly reversible, finely tuned regulatory enzyme system with which low concentrations of a hormone can exert large effects in rapidly reversible fashion, a characteristic of hormone action in vivo. Based on these findings, a new theory of hormone action has been developed and is termed "concerted theory of hormone and nucleotide action". Since a number of adenylate cyclase systems, stimulated by other hormones, seem to require nucleotides such as GTP for hormonal activation, it appears that this theory is generally applicable. For example, studies of ACTH action on the adenylate cyclase system in bovine or rat adrenals, have shown that GTP is essential for hormone action even in the presence of relatively high concentrations of substrate (0.5 mM to 1.0 mM ATP).

Recent evidence indicates that a metal ion, probably of the transition series, plays a crucial role in the regulation of adenylate cyclase activity. Chelators such as EGTA or EDTA, at concentrations as low as 10^{-11} M, stimulate both the glucagon- and ACTH-sensitive systems provided that GTP is present. At chelator concentrations of about 10^{-5} M in the presence of 10^{-5} M GTP, activation of these systems occurs so that hormone addition gives only slight further enhancement of activity. Histidine or cysteine, at concentrations of 10 to 20 mM also exert stimulatory effects. The effects of histidine are of interest since the NH_2-terminal histidine residue of glucagon is required for glucagon action. Des-1-his-glucagon binds competitively with glucagon at its receptor but does not activate adenylate cyclase. It is possible that the histidine residue interacts with the putative metal ion resulting in enhanced reactivity of GTP with its regulatory site.

A new mechanism of regulatory control of adenylate cyclase has recently been discovered which is observed only with the multi-receptor adenylate cyclase system in fat cells of the rat. GTP plays two distinct roles in regulating the activity and response of the enzyme to glucagon, secretin, epinephrine, ACTH, and TSH. A GTP-specific transferase enzyme in the plasma membrane appears to catalyse the phosphorylation of the enzyme component of the adenylate cyclase system. The enzyme also uses ITP though less efficiently. Phosphorylation of adenylate cyclase results in marked decreases in adenylate cyclase activity in the absence or presence of the hormones. When GTP is exhausted, restoration of normal activities occurs, suggesting that a phosphatase in the membranes causes enzymes dephosphorylation. The properties of the GTP-transferase enzyme and the phosphatase remain unknown. Recent studies have revealed that the fat cell contains a GTP-specific protein kinase that requires phosphorylation by a cyclic AMP-dependent protein kinase for its activity. The GTP-kinase is also present in liver and muscle and may prove to be an important regulatory enzyme. It is not involved in the regulation of fat and glycogen but may be responsible for the phosphorylation of adenylate cyclase.

Basal activity in fat cells is very high in contrast to that in most other types of cells and could, if not controlled, result in sufficient production of cyclic AMP to cause changes in the metabolism of the cell in the absence of hormones. It is conceivable that additional factors, including hormones other than those that stimulate adenylate cyclase, may regulate the activity of the phosphorylating enzyme (or phosphatase). Potential hormone candidates known to alter cyclic AMP production in fat cells and which do not exert direct effects on adenylate cyclase include such diverse agents as thyroxine, growth hormone, glucocorticoids, insulin, and prolactin.

The second role of GTP observed with the fat cell adenylate cyclase system is the same as that observed with the glucagon-sensitive system in hepatic membranes; i.e., GTP is required for the actions of the various hormones acting on the fat cell system. Thus, while basal activity (absence of hormones) is markedly reduced due to the phosphorylation mechanism described above, GTP also permits the expression of hormone action. The net result of the two effects of GTP is that, though total activity of the enzyme is reduced, the system becomes sensitive to the hormones.

It is clear that GTP plays a crucial role in hormonal activation of adenylate cyclase systems and in the regulation of the basal state of the fat cell enzyme system. The concerted actions of both hormone and GTP are required for activation of adenylate cyclase, a finding which suggests that cellular control of GTP metabolism may play a crucial role in the actions of hormones on the enzyme system and may explain certain disease states in which little hormone activity is observed in spite of high levels of circulating hormones. (M. Rodbell, M. C. Lin, Y. Salomon, B. Rubalcava, C. D. London and J. P. Harwood).

SECTION ON NUTRITIONAL BIOCHEMISTRY.

Metabolism of Fat-soluble Vitamins.

Studies of the metabolism of γ-tocopherol have shown that it has 9% of the vitamin E activity of α-tocopherol and that it accumulates in the tissues as does γ-tocopherol, but to a lesser extent. When α- and γ-tocopherols are fed together, the proportion of the two compounds in tissues vary widely. The reported involvement of γ-tocopherol in heme synthesis in rat liver could not be confirmed. (J. G. Bieri and R. Poukka-Evarts).

Collaborative studies with NINDS (Dr. W. T. London) sought to establish the minimal protein and energy requirements of pregnant rhesus monkeys. It was found that 2.1 grams of protein and 88 kilocalories per kilogram of body weight permitted normal maternal weight gain and produced infants of normal weight and appearance. (J. G. Bieri)

Net Uptake and Metabolism of Plasma Glutamine by Intestine.

The isolated, perfused rat intestine has been found to have a large uptake of glutamine, but of no other amino acid. This utilization of glutamine, which is 65% of the molar plasma glucose uptake, is apparently present in a variety of species including the monkey. In the germfree rat, glutamine nitrogen taken up by the gut can be accounted for in the blood as citrulline, alanine, ammonia and proline. The metabolism of glutamine occurs largely in cells of the small intestinal mucosa via glutamic acid and is not inhibited by high concentrations of glutamic acid. The concentration of glutamine in rat intestinal mucosa is much lower than that of other tissues. Evidence indicates that the gut mucosa may require exogenous glutamine for a normal rate of protein synthesis. (H. G. Windmueller).

Biochemical Studies of Cytochromes

Continuing studies of less dense sub-mitochondrial particles from rat liver have shown that the specific activities of cytochrome oxidase and monoamine oxidase are the same as in the more dense mitochondrial subfractions. Adenylate kinase activity was markedly lower while cytochrome c was more concentrated in the less dense particles. Adenylate kinase and NADH oxidase activities, and respiratory control, were similar in the two subfractions.

Studies of the effects of hormones on mitochondrial cytochrome c have shown that injected cortisol or testosterone did not alter this component. Injections of glucagen or allylisopropyl acetamide (an enzyme inducer) simply had little effect on mitochondrial cytochrome c concentration. However, when injected together, there was a significant increase.

SECTION ON VITAMIN METABOLISM

Dihydrofolic Acid Reductase

Purification of both bovine and chicken liver dihydrofolic reductase by affinity chromatography has been greatly facilitated by extraction from liver by homogenizing with 0.1 M $MnCl_2$. Treatment of the clarified extract with $ZnSO_4$ at pH 5 and then raising the pH to 8 yields an extract, after centrifugation, which can be directly absorbed by the methotrexate-Sepharose affinity system. Approximately 100 mg of "pure" dihydrofolic reductase has been isolated by this procedure from 12 kg of chicken liver.

The "pure" chicken liver enzyme exhibits a typical protein spectrum below 300 mμ. However, a small peak is observed at 345 mμ which suggests that TPNH is strongly bound to the enzyme, since extensive dialysis or gel filtration did not alter the spectrum. From a consideration of the binding of TPNH to the enzyme by kinetic analysis ($Km = 10^{-6}$ M), it is concluded that this tightly bound pyridine nucleotide represents a second molecule of TPNH bound to a site other than the active site (B.T. Kaufman).

Studies on Folic Acid

Attempts to degrade the polyglutamate derivative of N^5-methyl-tetrahydrofolic acid isolated from yeast by alkaline permanganate oxidation did not result in significant yields of the expected p-aminobenzoyl heptaglutamate. However, treating the initial compound with platinum oxide resulted in its conversion to a "dihydro" derivative which split into the expected pteridine and p-amino benzoyl polyglutamate upon acidification. Approximately 50% conversion to the p-amino benzoyl derivative was obtained by this procedure followed by chromatography on DEAE-cellulose with an ammonium acetate gradient.

In order to convert the N^5-methyl tetrahydrofolyl polyglutamate to the dimethylated tetrahydrofolic acid derivative, an attempt was made to transfer the methyl group to homocysteine via methionine biosynthesis. Thus, incubation of the yeast folate with homocysteine in the presence of a dialyzed yeast extract resulted in the slow appearance of a folate active for S. faecalis, which is a characteristic of tetrahydrofolic acid. These preliminary results suggest that yeast contains an enzyme which can carry out a direct transmethylation from the $\underline{N}^5$-methyl polyglutamate to homocysteine, since partial hydrolysis of the polyglutamate by "conjugase" treatment id not facilitate the rate of transmethylation. (B.T. Kaufman).

Properties of Folic Acid γ-Glutamyl Carboxypeptidase

In order to facilitate the isolation of the enzyme "folate conjugase", a rapid assay for enzymatic activity was developed. The standard assay which measures the amount of bacterial growth supported by the sample to be assayed, is very time consuming and requires an overnight incubation period before the results can be evaluated. In the assay, a conjugase reaction mixture is subjected to DEAE-cellulose thin layer chromatography. The folate used as substrate is cleanly separated from the product of digestion, which has fewer glutamyl residues. Semi-quantitative results obtained by visual estimation of the fluorescent intensities of the folate spots following spraying with appropriate reagents are sufficiently accurate for assay of column fractions. Excellent quantitation can be obtained by fluorescence scanning of the chromatograms. Using the DEAE-cellulose thin-layer chromatographic technique and the major folates from yeast and Pediococcus cerevisiae, it has been shown that the conjugases from chick liver and pancreas differ both in the final product of their hydrolysis and in the production of intermediate forms during the reaction.

Both for use as a substrate in folate conjugase assays and because of its intrinsic interest, the large-scale purification of the major folate from P. cerevisiae has been undertaken. Earlier studies on this folate suggested that it had four glutamyl residues, making it the first folate with four glutamic acid units isolated from a natural source. Furthermore, its $\underline{N}^5$ formyl tetrahydro-pteridine ring structure, imparting considerable stability to the molecule, in contrast to the instability of many reduced folates, favors the use of P. cerevisiae as a substrate source for folate conjugase. (G. Reeck and H. Bakerman).

COLLABORATIVE RESEARCH

Effects of Hormone Metabolism of Adipose Tissue Perfused *in vivo*:-
with Dr. Vance Kovacev, Department of Physiology, Medical Faculty, University of Skopje, Skopje, Macedonia, Yugoslavia (S. S. Chernick)

Ultrastructural Aspects of Lipid Metabolism: -
with Dr. Olga Stein, Department of Experimental Medicine and Cancer Research, Hebrew University-Hadassah Medical School, Jerusalem, Israel (R. O. Scow)

Control of Lipid Synthesis by the Liver:-
with Dr. Benyamin Shapiro, Department of Biochemistry, Hebrew University, Hadassah Medical School, Jerusalem, Israel (M. Rodbell)

Metabolic Studies of Vitamin A:-
with Dr. J. Ganguly, Department of Biochemistry, Indian Institute of Science, Bangalore, India (J. Bieri)

Effect of DNA and DNA Nucleotides in the Reaction of the Immune Response:-
with Dr. Michael Feldman, Department of Cell Biology, Weizmann Institute of Science, Rehovoth, Israel (H. A. Sober)

A Study of Individual Oligopeptide Members of Polyamino Acid Series:-
with Dr. Ephraim Katchalski, Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel (H. A. Sober)

Serial No. NIAMDD-LNE-1
1. Nutrition and Endocrinology
2. Vitamin Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on folic acid.

Previous Serial Number: SAME

Principal Investigator: Dr. B. T. Kaufman

Other Investigators: Miss R. C. Gardiner and Mrs. M. K. Romine

Cooperating Units: None

Man Years

Total:	4.0
Professional:	1.0
Other:	3.0

Project Description:

Objectives: To isolate and determine the biochemical properties of enzymes concerned with the metabolism of folic acid, especially dihydrofolic reductase from chicken liver, yeast, bacteria and certain leukemic tumor cells. To study the interaction of these enzymes with agents known to result in activation, as well as certain anti-leukemic drugs which result in inactivation. To isolate and determine the chemical and biological properties of the naturally occurring forms of folic acid in various cells and tissues.

Methods Employed: Standard chemical and biochemical techniques appropriate for purification and fractionation of vitamins and proteins are utilized in these projects in conjunction with various methods of enzymatic and microbiological analyses.

Major Findings: Due to the very small amounts of dihydrofolic reductase present in liver (about 10 mg/kg), the isolation of this enzyme by conventional technqiues is quite laborious. The affinity column previously described for the isolation of dihydrofolic reductase was shown to be effective for the purification of small amounts of enzyme (2 to 5 mg) from chicken liver. However, several problems precluded the routine use of this system for large scale preparations. Thus, despite the high degree of specificity methotrexate exhibits as an inhibitor of dihydrofolic reductase, the affinity column prepared from this folate antagonist binds large amounts of extraneous protein. In fact, some of these materials appear to bind to the methotrexate column more strongly than the enzyme itself. Therefore, it became necessary to partially purify the crude liver extract by two gel filtration columns before using the affinity column.

The gel filtration steps can be avoided by extraction of the tissue with MnCl2 and treatment of the crude extract with $ZnSO_4$ at pH 5 and subsequently raising the pH to 8 to produce a zinc hydroxide gel. Although the enzyme is temporarily inactivated by this procedure, full recovery of activity is obtained by treatment with EDTA and precipitation of the protein with ammonium sulfate. The resuspended precipitate can then be directly absorbed on to the affinity column. Furthermore, the capacity of the column to continuously absorb activity from subsequent additions of sample is maintained by washing the column with 5 M urea after each addition of crude extract. Under these conditions, approximately 90% of dihydrofolic reductase has been absorbed from 15 kilos of liver by adding the extract from 1 kilo at a time to a column (25 x 100 mm) over a period of 30 days. A recovery of 120 mg of enzyme was obtained. It is of interest, however, that this represents only 0.1% of the hypothetical binding of the column.

Although the material eluted from the affinity column appears to be homogeneous, the specific activity is only 80% of theory. For example, a single protein peak is obtained by both chromatography on Sephadex G-100 and hydroxylapatite. In each case, the specific activity is essentially constant across the chromatograms. However, if a large excess of TPNH is added to the preparation which is then rechromatographed on hydroxylapatite, two peaks of protein are now obtained; the major peak exhibiting the theoretical specific activity but a small peak with very low specific activity is also seen. However, both peaks contain bound TPNH which could not be removed by precipitation with ammonium sulfate and exhaustive dialysis. It will be necessary to remove this bound substrate in order to study the proposed changes in the conformation and activation of the enzyme as a function of substrate binding.

Attempts to degrade the polyglutamate derivative of N^5-methyltetrahydrofolic acid, isolated from yeast, by alkaline permanganate oxidation did not result in significant yields of the expected p-aminobenzoyl heptaglutamate. However, treating the initial compound with platinum oxide resulted in its conversion to a dihydro- derivative which split into the expected pteridine and p-amino benzoyl polyglutamate upon acidification. Approximately 50% conversion to the p-amino benzoyl derivative was obtained by this procedure followed by chromatography on DEAE-cellulose with an ammonium acetate gradient.

In order to convert the N^5-methyl tetrahydrofolyl polyglutamate to the demethylated tetrahydrofolic acid derivative, an attempt was made to transfer the methyl group to homocysteine via methionine biosynthesis. Thus, incubation of the yeast folate with homocysteine in the presence of a dialyzed yeast extract resulted in the slow appearance of a folate active for *S. faecalis*, which is characteristic of tetrahydrofolic acid. These preliminary results suggest that yeast contains an enzyme which can carry out a direct transmethylation from ththe N^5-methyl polyglutamate to homocysteine since partial hydrolysis of the polyglutamate by "conjugase" treatment did not facilitate the rate of transmethylation.

Significance to Bio-Medical Research and the Program of the Institute: Dihydrofolic reductase is not only a key enzyme in the metabolism of folic acid, it is also of major importance in the synthesis of thymidine and the chief site of action of the anti-folic drugs. Thus, the understanding of the structure and function of this enzyme would be of basic importance in both normal and mebolic interrelationships and in cancer chemotherapy. Folic acid, as such, most probably does not occur in nature. Thus, the knowledge of chemical structure and biological properties of the more complex natural form will result in a better understanding of the biochemistry of this vitamin.

Proposed Course of Project: Investigations will continue on the molecular properties and the mechanism of action of liver dihydrofolic reductase with particular emphasis on the nature of mercury and iodine activation and methotrexate inhibition. Attempts will be made to convert the naturally occurring complex folate from yeast into other cofactor forms of folic acid, such as the tetrahydro, dihydro, and the fully oxidized forms.

Honors and Awards: None

Publications: None.

Serial No. NIAMDD-LNE-2
1. Nutrition and Endocrinology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Large-scale processing of biological materials.

Previous Serial Number: SAME

Principal Investigator: Mr. D. L. Rogerson, Jr.

Other Investigator: Dr. H. A. Sober

Cooperating Units: None

Man Years

Total:	3.0
Professional:	1.0
Other:	2.0

Project Description:

Objectives: Large amounts of natural materials, such as bacteria, animal tissues, cells, blood, urine and plant materials require facilities for their production and processing so that they may be studied under standard laboratory conditions. Facilities for such large-scale processing are provided under this project.

Methods Employed: When called upon for large-scale production by any NIH investigator, methods are designed for the scaling up of small laboratory experiments utilizing large reaction vessels, extractors, stills, concentrators, centrifuges, etc., so that the processing can be carried out effectively, efficiently and safely. Two 10-liter, one 50-liter, two 300-liter and one 1100-liter fermentorsare utilized for growing microorganisms under a variety of conditions. Bacterial cells and yeasts are harvested by means of laboratory or large-scale AS-16P centrifuges. Protozoa are concentrated in continuous flow centrifuges. Cells are either supplied as a paste or further processed to yield cell-free suspensions by rupture with a Gaulin homogenizer. In some instances, a specific substance is isolated and partially purified by further processing of the culture supernatant or the cell paste. A high capacity, Model AS-26P Sharples centrifuge is now on order to facilitate the recovery of microorganisms from the 1100-liter fermentor.

Major Findings: During the past year, 252 requests by NIH investigators were processed. 221 kilogram quantities (54,600 liters of cultures) of 16 microorganisms were produced. The microorganisms grown during this period included bacteria and yeast and consisted of wild type and mutant strains of Aerobacter aerogenes, Bacillus amyloliquefaciens, Bacillus subtilis, Citrobacter sp, Corynebacterium acnes, Comomonas sp, Erwinia cartovora, Escherichia coli, Haemophilus influenzae, Haemophilus parainfluenzae, Pediococcus cerevisiae, Pseudomonas sp., Saccharomyces cerevisiae, Salmonella typhimurium, Streptococcus lactis and Streptococcus mutans. Some of the organisms were used as hosts for bacteriophage production, such as fd, MS2, Φ 80, lambda, R17 and T4. Others, such as Bacillus amyloliquefaciens, were used to produce extracellular enzymes. Microorganisms were grown 86 times in the 300-liter, 38 times in the 10-liter, 24 times in the 1100-liter fermentor and 41 times in the 50-liter fermentors.

Studies were continued with the NICHD for polysaccharide production by Haemophilus influenzae and cross-reacting organisms such as Escherichia coli. Also a polysaccharide fraction containing the protective antigen of typhoid fever was isolated from a non-pathogenic organism, Citrobacter sp. E. coli W6 spheroplasts were produced for the NHLI three times, using 10-, 50-, and 300-liter fermentors for each preparation. The Gaulin laboratory homogenizer was used 47 times to rupture suspensions of yeast or bacterial cells. Large-scale processing activities consisted of the preparation of Histamine-N-methyltransferase enzyme from 1.8 kg guinea pig brains (repeated with 2.6 kg), the extraction of hormones from parathyroid substance in six, one kilogram preparations, the isolation of an inhibitor, barstar, from 15 kg Bacillus amyloliquefaciens, the preparation (twice) of Deoxyribonucleotide polymerizing enzyme from 10 lbs calf thymus glands, the isolation and chromatographic purification of histidase from 2 kg of a pseudomonad ATCC 11299b, the processing of 5.8 kgm Koelreutria paniculata seeds for an alcohol-soluble substance and isolation of rennin from 20 lbs hog kidney. The KII Zonal centrifuge as used to band cell membranes from bovine thyroid glands, and for the separation of E. coli J5 membranes. Efforts were made to separate and purify red blood cell "ghosts". The large capacity and turbine Sharples centrifuges were used to separate cells from suspensions such as 15 liters Basidiomycetes, Lactobacillus fructosus and red blood cell "ghosts" from 30 units of hemolyzed human red blood cells. Various quantities of plant and animal tissues such as bovine tendon and calf muscles were ground in the assorted laboratory mills.

Significance to Bio-Medical Research and the Program of the Institute:

With the laboratory's facilities many projects can be assisted where large amounts of natural material can be produced and processed.

Proposed Course of Project: To continue assisting NIH investigators when large-scale processing of biological materials is needed in their projects and to expand the facilities for isolation and purification of biologically-important macromolecules, cell particulates and materials.

Honors and Awards: NONE

Publications:

Hartley, R. W. and Rogerson, D. L.Jr.,: Production and purification of the extra-cellular ribonuclease of Bacillus amyloliquefaciens (Barnase) and its

intra-cellular inhibitor (Barstar). I. BARNASE. Preparative Biochemistry, 2: 229-242, 1972.

Hartley, R.W., Rogerson, D. L. Jr., and Smeaton, J. R.: Production and purification of the extracellular ribonuclease of *Bacillus amyloliquefaciens* (Barnase) and its intracellular inhibitor (Barstar). II. BARSTAR. Preparative Biochemistry, 2: 243-250, 1972.

Serial No. NIAMDD-LNE-3
1. Nutrition and Endocrinology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies in experimental nutrition.

Previous Serial Number: Same

Principal Investigator: Mr. E. G. McDaniel

Other Investigators:
Dr. F. S. Doft, Guest Worker (LNE, NIAMDD)
Dr. G. Laqueur (LEP-NIAMDD)
Dr. J. Smith (VA Hospital, Trace Element Lab).

Cooperating Units: None

Man Years:

Total:	2.0
Professional:	1.0
Other:	1.0

Project Descrioption:

Objectives: To determine the nutritional, biochemical and physiological characteristics of germfree and conventional laboratory animals.

Methods Employed: Germfree and conventional rats and chicks are maintained on diets of known composition. Growth, blood picture and other physiological functions are studied. Germfree animals are inoculated with known strains of bacterium to determine the organisms responsible for various phenomena. Conventional and/or ex-germfree animals are used in conjunction with germfree work.

Major Findings: With Dr. Laqueur, studies of the toxic effect of cycasin have been continued. Cycasin is not toxic to adult germfree rats but is toxic to conventional rats, to very young germfree rats and to ex-germfree rats infected with bacteria which are capable of converting the compound to its aglycone. Previously germfree rats, infected with bacteria which are unable to make this conversion, react like germfree rats and show no symptoms of toxicity to cycasin. The aglycone of cycasin is toxic to germfree as well as conventional rats at any age. Although adult rats are incapable of converting cycasin to aglycone in the absence of bacteria, the very young animal is able to do so but this capability is lost early in life. Experiments have been done to determine when the animal loses the ability to make this conversion within its own tissue and without the presence of bacteria. Single doses of cycasin were administered to germfree rats at five, nine, sixteen, twenty-five and thirty-five days of age. Results indicate that at some point between sixteen and twenty-five days of age, the ability of the germfree rat to convert cycasin to the toxic aglycone is sharply reduced, although some tumors were observed in animals which had received cycasin as late as 35 days of age.

With Dr. Smith, a series of experiments have been done 1) to study development, prevention and treatment of deficiencies of the fat soluble Vitamin A and the trace mineral zinc, 2) to try to identify some of the functions and to study possible interrelationships between these two dissimilar dietary factors, 3) to study the influence of bacteria upon the functions and requirements of these factors. It has been noted that bacteria have a marked influence upon development and severity of deficiency symptoms, survival time and perhaps upon the actual requirement of both vitamin A and zinc. In both cases, it appears that as progressively lower levels of these factors are attained, the differences observed between germfree and conventional animals, although still evident, becomes less marked. This suggests that the differences observed may result from a more efficient utilization by the germfree animals or from competition by the bacteria for nutritional factors. When rats were fed diets containing borderline levels of zinc, conventional or ex-germfree animals required a higher level of the mineral to attain growth equal to that of the germfree rats.

In rats subjected to a double deficiency of vitamin A and zinc, it has been possible to precipitate a very early and acute deficiency state by treating the doubly deficient animals with zinc alone. This apparent vitamin A deficiency is characterized by crippling which is much more severe and occurs much earlier than that caused by depletion of vitamin A alone. The symptoms occur after a short period of rapid growth which follows the administration of zinc. It is possible that this period of rapid growth depletes the stores of vitamin A. Since animals made A-deficient by withholding A alone grow to a much larger size and appear to retain their vitamin A stores much longer, the period of zinc deficiency must have altered their nutritional status with respect to vitamin A. The crippling observed in vitamin A deficiency responds to treatment with vitamin A, but recovery is not always complete, suggesting that some irreversible damage may result from the severe A deficiency. This is true in rats which have been subjected to vitamin A deficiency alone as well as those which have also been zinc deficient. Tissues from effected rats are being studied histologically to try to identify specific lesions.

Rats subjected to the double deficiency for four weeks exhibit abnormally low levels of vitamin A in both liver and blood. Treatment with vitamin A alone resulted in the accumulation of large amounts of the vitamin in the liver but with below normal levels in the plasma, while treatment with both vitamin A and zinc resulted in more nearly normal levels of the vitamin in both liver and plasma. It was suspected that in a state of zinc deficiency the rat was unable to mobilize vitamin A from the liver in order to maintain normal blood levels. To test this further, rats were made doubly deficient for four weeks, then given vitamin A without zinc for an additional period of two weeks at which time the animals exhibited high liver A and lower than normal plasma A levels. Treatment with zinc was then started. At the end of an additional two week period, the liver A had decreased and the plasma A had increased to normal levels. Similar results were observed whether or not the administration of A was continued during the period of zinc treatment. These observations strongly suggest that zinc is necessary for the mobilization and transport of vitamin A from the liver stores to the blood and possibly to the sites of action in the body. It is further suggested that an animal which is zinc deficient may also, in effect, be vitamin A deficient even though sufficient vitamin A is in the diet.

When doubly deficient rats are treated, growth follows when zinc or zinc plus vitamin A are given, but not when A alone is given. Therefore, it was thought desirable to limit the growth of some zinc treated rats to that of rats treated with A alone. It was observed that when growth was restricted by limiting food intake during the period of zinc treatment, the animal appeared to be less able to mobilize the liver vitamin A. This finding was unexpected and is being further studied. It may indicate that the period of deficiency of vitamin A or zinc or both causes some irreversible damage which can be overcome only by permitting new tissue to grow.

Experiments in progress are designed to study the mechanism by which zinc functions in the mobilization and transport of vitamin A, the cause of the crippling observed in vitamin A deficiency and the influence of bacteria in the development and prevention of deficiencies of these dietary factors.

Significance to Bio-Medical Research and the Program of the Institute:

The work with germfree animals will give us a better understanding of the relationships between the animal's intestinal flora and its physiological reactions both to dietary nutrients and to other factors. The work with the germfree animals emphasizes the importance of nutrition in long-term studies.

Proposed Course of Project: Experiments will be conducted in an attempt to determine more precisely the relationship between the host animal and its bacterial flora with respect to its influence upon the nutrition and physiology of the host. Attempts will be made to identify the specific organisms which are responsible for such influence and, if possible, determine methods to counteract such influences as may be deleterious to the host.

Honors and Awards: None

Publications:

Smith, J. C. Jr., McDaniel, E. G., McBean, L.D., Doft, F.S., and Halstead, J. A.: Effect of micro-organisms upon zinc metaoblism using germfree and conventional rats. J. of Nutr. 102: 711-720, 1972.

Serial No. NIAMDD-LNE-4
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Study of protein hormones: Chemistry and physiological actions of protein hormones.

Previous Serial Number: NIAMD-LNE-4

Principal Investigator: Dr. Robert W. Bates

Other Investigators: None

Cooperating Units: None

Man Years

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives: To isolate protein hormones from the pituitary glands of man and animals, functional, transplantable mouse or rat pituitary tumors, and blood, especially the anterior pituitary hormones; to investigate the nature of these hormones as they circulate in the blood stream; to study the effect of hormones produced in excess by tumors on the host animal; to investigate the induction of diabetes by hormones.

Methods Employed: Hormones and other substances are extracted from pituitary gland, rat or mouse pituitary tumors, pancreas and blood. Crude extracts which may contain several hormones are fractionated by techniques commonly used in protein chemistry, such as salt precipitation, solvent fractionation, iso-electric precipitation, preparative and disc electrophoresis, ion-exchange chromatography, and gel filtration. Various quantitative biological assays are required to determine the concentration of the several hormones in tissues and protein fractions. Radioimmunoassay techniques are employed to extend the studies to concentration levels beyond the reach of conventional bioassay procedures. Removal of various endocrine glands with and without hormonal replacement is a critical technique. Effects of elimination or modification of hormonal production of the thyroid by antithyroid drugs and of the adrenal cortex by anti-adrenal drugs is compared to effects of surgical removal of the gland.

Major Findings: Hormonal Induction of Diabetes with Exogenous Hormones: Most of our studies this year have been concerned with changes that occur in levels of blood glucose, serum insulin and pancreatic insulin in rats injected three times daily with diabetogenic hormones. Some of the rats were also fed 0.5% tolbutamide in the diet. When possible, the dosages used were large enough to produce glucosuria in 3-5 days.

Serum insulin in rats fed tolbutamide increased about 50% for only 6-8 hours, returned to normal by 24 hours, and remained there for up to 5 days. Blood glucose decreased 20-30 mg/100 ml at the same time that serum insulin was elevated, and then returned towards normal by days 1-5 while still on the tolbutamide diet. In contrast, the pancreatic insulin concentration fell to 30% of normal by day 4 and then began to recover on day 5. Thus the main effect of tolbutamide was to lower pancreatic insulin level (insulin reserve).

Bovine growth hormone (BGH) increased serum insulin by 24 hours but did not alter blood glucose. Pancreatic insulin concentration gradually increased about 50% by 5 days. This was the main action of BGH.

Cortisol and dexamethasone caused a rapid 3-4 fold rise in serum insulin level during the first 24 hour period and maintained this high level, usually, for 5 days. In spite of the high serum insulin level there was little change in blood sugar during the first 24 hours. During subsequent days there was a rise in blood sugar to over 200 mg/100 ml with glucosuria by day 3-4. Pancreatic insulin changed little during the first two days and fell thereafter to about 50% of normal.

Synergistic combinations of BGH with cortisol or dexamethasone produced similar but larger changes than did the individual adrenal hormones. These changes were further magnified if tolbutamide was fed in the diet. The main action of tolbutamide was, again, to decrease insulin concentration in the pancreas. Without exception, and even with tolbutamide in the diet, there was a uniform return toward normal of the pancreatic insulin concentration by day 5 with hormonal treatment. In one group injected with BGH and cortisol for 14 days, the pancreatic insulin concentration increased from a level of 0.7 U/g on day 4 to a normal level of 2 U/g on day 14. These rats had at day 14 elevated serum insulin levels (80 μU/mg, 2 x normal), high blood glucose levels (255 mg/100 ml), and glucosuria (4 g/day).

These data demonstrate the great capacity of the rat to maintain the insulin concentration in the pancreas and, especially, the ability of the β cells to produce additional insulin in response to persistent hormonal treatment. Tolbutamide reduced by a factor of about 2 the dosage necessary to induce glucosuria in all hormonal treatments. It also reduced the time of appearance of glucosuria from about 4 days to 2 days and decreased by 60-75% the concentration of insulin in the pancreas at day 5. These three effects of tolbutamide are all in the direction of diabetogenesis.

Similar studies in rats with transplantable hormone producing pituitary tumors (MtT) have been continued. These chronically endogenously stimulated rats have normal blood glucose levels (and no glucosuria) with serum insulin levels 4-5 times normal and pancreatic insulin levels 3-4 times normal. This shows again the ability of the β cells in the rat to produce insulin, perhaps, as a direct result of hormonal stimuli.

These studies show that during the first day diabetogenic hormones increase plasma insulin levels with little change in blood sugar or pancreatic insulin levels. Subsequently, with continued treatment, the pancreatic insulin levels fall (especially with tolbutamide added), and hyperglycemia and glucosuria develop in the presence of the elevated serum insulin levels. This suggests that the hormones have a stimulatory effect on insulin release from the pancreas initially followed by more slowly developing systemic actions which result in the development of hyperglycemia, which in turn stimulates release of insulin.

Effect of Hormones on Organ Weights: Liver, kidney, adrenal, thymus, pancreas and depot fat were weighed routinely when the rats on our diabetes studies were killed. These data have not all been analyzed. Certain old, well established observations, such as atrophy of thymus and adrenal with glucocorticoids, were made. Kidney weight increased progressively, as much as 50% in 5 days, with time and with dose of glucocorticoids or ACTH. In the case of ACTH treatment, adrenal weight and kidney weight increase were coordinated confirming our earlier studies with rats with tumors which showed the correlation of kidney and adrenal weights. There were differences in the adrenal weight response to the type of formulation of the ACTH (gel or Zn) and to simultaneous treatment with other hormones. That is to say that hormonal interactions on adrenal and kidney weight were found.

Weight of depot fat was not appreciably changed in five days. Liver weight often increased significantly in 24 hours but with little subsequent change unlike kidney weight. Pancreas weight varied primarily with the food intake, as previously reported.

The hormonal control of metabolism and body and organ size becomes more impressive with each experiment. The interaction of the many hormones, which fluctuates with time and dosage, makes these endocrine studies difficult to interpret and endless in number but immensely important.

Significance to Bio-medical Research and the Program of the Institute: One of the major roles of the six hormones of the anterior pituitary gland is to modify or regulate the size and functional activity of other glands or tissues, which in turn control the overall metabolic state of the individual. This hormonal balance or imbalance controls how one feels and what one can do. Certain metabolic abnormalities and tumors are corrected by treatment with hormones or by removal of endocrine glands. Purified hormone preparations are needed for this work together with sensitive and quantitative tests which will estimate the concentration of hormones in the blood or transport system and the rate of release of the hormones. One needs to know what factors control the biosynthesis and subsequent release of hormones by the pituitary gland.

The studies on induction of diabetes reveal the major role of hormonal interaction in a synergistic manner of at least three hormones or types of hormones. The extent of this synergism is such that hormonal induction of diabetes in man should be seriously investigated. The use of tolbutamide as an antidiabetic agent is contraindicated by our studies so far.

Proposed Course: Studies on the induction of diabetes by hormones in rats that are 80% pancreatectomized will be carried out in a systematic manner to throw light on the problem of hormonal interaction (potentiation or inhibition) and to assess the significance of hormonal balance.

Analysis of hormone levels in rats with transplantable pituitary tumors will continue. A search for factors which modify these levels will be made. The physiological action of the hormones from the pituitary tumors upon the host will be studied. Studies on the changes occurring after tumor removal are planned.

The question of identity of human GH and prolactin results in confusion when one attempts to establish potency of prolactin in plasma of humans in various clinical conditions. Also the specificity of the cropsac response in pigeons raises the question of whether the response obtained with urine and plasma extracts is due to prolactin. These problems are being investigated.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LNE-5
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Diabetes and Fat Metabolism

Previous Serial Number: NIAMD-LNE-5

Principal Investigators: Drs. Robert O. Scow and Sidney S. Chernick

Other Investigators:
Dr. Margit Hamosh, Visiting Scientist
Dr. Carole R. Mendelson, Guest Worker
Dr. Oren Zinder, Guest Worker
Dr. Cecilio Linder, Guest Worker
Dr. Hayden Klaeveman (NIAMDD-D & HD)

Cooperating Units:
NIAMDD-LNE-6
NIAMDD-D & HD

PL 480 Agreement No. 02-009-1 with the
Department of Physiology, University of Skopje
Skopje, Yugoslavia

Man Years:

Total: 6½
Professional: 2½
Other: 4

Project Description:

Objectives: To determine influence of hormones and other factors on metabolism of fat and carbohydrate in liver, adipose tissue and mammary gland of normal and diabetic animals.

Methods Employed: Animals are deprived of one or several endocrine glands and treated with hormones or other substances. The effect of the above on the metabolism of fat and carbohydrate in liver, adipose tissue and other organs and on blood hormone levels are determined with *in vivo* and *in vitro* studies using conventional and radioisotope techniques, and electron microscopy. Effects of hormones added *in vitro* are also studied.

Major Findings: Metabolism of chylomicron-triglyceride by perfused rat mammary gland: Studies reported last year showed that chylomicron-triglyceride is taken up and hydrolyzed by perfused lactating rat mammary gland, and that uptake and hydrolysis were both decreased 80% when the rats were unsuckled for 18 hours prior to experiment. Other studies had shown that lipoprotein lipase activity in mammary gland is greatly increased by lactation, and decreased by nonsuckling. Additional evidence that the enzyme is involved in uptake of blood triglyceride was obtained in perfused mammary tissue of hypophysectomized lactating rats; uptake and hydrolysis of triglyceride as well as lipoprotein lipase activity were negligible. Perfused mammary gland of lactating rats also hydrolyzed triglyceride in an artificial emulsion (Intralipid) but only if the emulsion had been previously activated by incubating it with rat serum. Activation prior to perfusion was necessary since the perfusing fluid used in these studies was serum-free and, thus, did not contain the lipoprotein needed to make triglyceride in the emulsion accessible to the lipolytic action of the enzyme.

Since studies with doubly labeled chylomicron-triglyceride (^{14}C-palmitate and ^{3}H-glycerol) showed that glyceride-glycerol was retained by the tissue, it was of interest to determine if glycerol, one of the products of hydrolysis, could be utilized by perfused mammary gland. It was found that 18% of glycerol infused at a blood concentration of 0.6 mM was incorporated into triglyceride, indicating the presence of an active glycerokinase in mammary gland of lactating rats. The high molar ratio of free fatty acids (FFA) to glycerol, > 5, found in venous blood when chylomicrons were infused indicates that partial glycerides are formed during the initial step of triglyceride uptake. Further studies are necessary to determine if partial glycerides can be utilized, without further hydrolysis, by mammary alveolar cells.

Lipoprotein lipase activity and uptake of blood triglyceride by red and white skeletal muscle: Studies in several tissues (adipose, mammary, heart and diaphragm muscle) have shown that uptake of blood triglyceride is related to the level of lipoprotein lipase activity in the tissues. Skeletal muscle also utilizes circulating triglyceride for energy, but the possible role of lipoprotein lipase in this tissue has not been evaluated. Therefore, the relationship between lipoprotein lipase activity and triglyceride uptake were studied in red and white muscle of the rat. The effects of fasting were also investigated, since fasting increases lipoprotein lipase activity and triglyceride uptake in heart and diaphragm muscle, and decreases both in adipose tissue.

It was found that lipoprotein lipase activity is higher in red skeletal muscle (soleus, medial head of triceps brachii and posterior belly of digastric) than in white muscle (abdominal, pectoralis, psoas minor, tibialis anterior, and long head of triceps brachii), and that fasting 1-2 days increased the activity in red but not in white muscle. The level of lipoprotein lipase activity in the tissues was closely related to uptake of blood triglyceride by the muscles in vivo, providing evidence that the enzyme is also involved in triglyceride uptake in skeletal muscle. The higher uptake by red muscle, especially during fasting, may be related to its greater dependence on fatty acids for energy.

Lingual lipase: Earlier studies in this laboratory showed that lingual serous glands in the rat secrete a very potent lipase which hydrolyzes dietary triglyceride to mostly diglyceride and FFA at pH 4.5-5.5 and that this enzyme accounts for most of the lipolytic activity found in the stomach. A similar activity has been found in human in aspirates from the middle and lower parts of the esophagus and from the stomach. Studies are in progress to determine the origin of the enzyme and its role in digestion of dietary lipid in man.

Significance to Biomedical Research in the Program of the Institute: Uptake of blood triglycerides by most tissues involves hydrolysis of triglyceride to FFA and glyceride by lipoprotein lipase. Cytochemical studies from this laboratory showed that hydrolysis occurs in the capillary endothelium and basement membrane in adipose tissue. Findings in perfused adipose tissue suggest that hydrolysis of triglyceride occurs stepwise, with partial glycerides formed at the luminal surface and hydrolysis completed in the basement membrane area. Studies in perfused mammary tissue also showed that partial glycerides are formed during uptake of blood triglyceride. It is possible that lipoprotein lipase in the capillary endothelium hydrolyzes triglyceride to FFA and monoglyceride and that another enzyme, perhaps from periendothelial cells (pericytes), hydrolyzes monoglyceride to FFA and glycerol. If uptake of triglyceride should involve two different enzymes, glyceride could accumulate in capillary endothelium whenever lipoprotein lipase was more active than monoglyceride lipase. Whether such a process occurs in capillaries or in the endothelium of larger vessels is being studied.

It was reported earlier that a lingual lipase had been discovered in rat which hydrolyzes triglyceride to partial glyceride (mostly diglyceride) and FFA at the pH (4.5-5.5) present in the stomach following a meal. Recent studies in humans show that a similar lipolytic activity is present in aspirates of the upper esophagus and stomach. Studies are in progress to determine the origin of the lipase in man. The findings, in man and rat, suggest that this lipase converts dietary triglyceride to diglyceride and free fatty acid in the stomach. Since fatty acids are not dissociated at pH 4.5-5.5, they probably are present in stomach in triglyceride-diglyceride droplets and thus exert no detergent effect until they enter the duodenum and become soaps. The role of this process in the emulsification and digestion of lipid by pancreatic lipase is under study.

Proposed Course: Adipose tissue will be perfused with emulsions of triglyceride containing labeled fatty acids in specific positions in order to determine more precisely where and how the triglyceride molecule is hydrolyzed after it is removed from the blood. Emulsions of partial glycerides will also be perfused to determine if they can be utilized by the tissue, and if so electron microscope cytochemical studies will be made to localize where in the capillary wall they are hydrolyzed.

Perfusion studies of lactating mammary tissue will be made to determine the form in which glyceride-fatty acids enter mammary epithelial cells. The large retention of chylomicron glyceride-glycerol in mammary tissues suggests that either partial glycerides or glycerol liberated from chylomicrons are utilized by the tissue. Incorporation of chylomicron fatty acids into milk lipid will also be studied.

Lingual lipase activity has been studied mostly in rats. Studies will be made to determine the source of the lipolytic activity found in esophageal aspirates of man. Studies will also be made to determine its role in lipid digestion and to determine what factors regulate its synthesis and secretion. Isolation and purification of the enzyme will be initiated as soon as an ample source can be found.

Honors and Awards: None

Publications:

Chernick, S.S., Clark, C.M., Gardiner, R.J. and Scow, R.O. Role of lipolytic and glucocorticoid hormones in the development of diabetic ketosis. Diabetes 21:946-954, 1972.

Hamosh, M. and Scow, R.O. Lingual lipase and its role in the digestion of dietary lipid. J. Clin. Invest. 52:88-95, 1973.

Hamosh, M. and Scow, R.O. Lingual lipase. Proceedings of IV Symposium on Oral Sensation and Perception: Development in the Fetus and Infant. In press.

Mendelson, C.R. and Scow, R.O. Uptake of chylomicron-triglyceride by perfused mammary tissue of lactating rats. Am. J. Physiol. 223:1418-1423, 1972.

Scow, R.O., Hamosh, M., Blanchette-Mackie, E.J. and Evans, A.J. Uptake of blood triglyceride by various tissues. Lipids 7:497-505, 1972.

Scow, R.O., Mendelson, C.R., Zinder, O., Hamosh, M. and Blanchette-Mackie, E.J. Role of lipoprotein lipase in the delivery of dietary fatty acids to lactating mammary tissue. In Galli, C., Jacini, G. and Pecile, A. (Eds.) Dietary Lipids and Postnatal Development. New York. Raven Press. In press.

Scow, R.O. (Ed.): Endocrinology, Proceedings of IV International Congress of Endocrinology. Amsterdam. Excerpta Medica. In press.

Serial No. NIAMDD-LNE-6
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Transport of lipid, hormones and enzymes through cells and membranes

Previous Serial Number: NIAMDD-LNE-6

Principal Investigators: Drs. E. Joan Blanchette-Mackie and Robert O. Scow

Other Investigators: Gloria DeVore, Guest Worker

Cooperating Unit: NIAMDD-LNE-5

PL 480 Project No. 06-101-1 with Department of Experimental Medicine and Cancer Research, Hebrew University-Hadassah Medical School, Jerusalem, Israel

Man Years:

Total:	2½
Professional:	1½
Other:	1

Project Description:

Objectives: To study the structure and metabolism of cells that synthesize and secrete plasma lipid and hormones. To study the transport of lipid, hormones and enzymes across the capillary endothelium, basement membrane and extracellular space in different kinds of tissues. To study the uptake of lipid, hormones and enzymes by different kinds of cells. To correlate structural and biochemical changes induced in cells and tissues by various means.

Methods Employed: Animals of different ages and in various physiological states (fasting, pregnant, lactating, diabetic) are used to study with biochemical and morphological techniques the synthesis and secretion of lipoprotein triglyceride by liver and intestines, and the utilization of plasma triglyceride by adipose tissue, mammary gland and muscle. Perfused adipose and mammary tissues are used to localize with cytochemical techniques the sites of action of lipoprotein lipase and the mode of transport of fatty acids between blood and tissue cells. Perfused tissues are also used to determine with immuno-cytochemical techniques the distribution and site of synthesis of lipoprotein lipase and the transport of hormones across capillary endothelium and extracellular space. Isolated chylomicrons and purified lipoprotein lipase incubated *in vitro* are used to study the morphological changes in chylomicrons induced when the triglyceride core is reduced by lipolysis.

Major Findings: Metabolism of chylomicrons in vitro: Chylomicrons are spheres consisting of a core of triglyceride, with traces of cholesterol ester, enclosed by a surface film of phospholipid, cholesterol and protein. Chylomicron-triglyceride is hydrolyzed to FFA and glycerol by lipoprotein lipase. Morphological studies with the electron microscope showed that FFA produced by hydrolysis of chylomicron-triglyceride accumulate within the chylomicron core when the FFA-binding sites on albumin are filled. Thus, albumin may have a specific role in the transfer of FFA across the chylomicron surface film. The studies also showed that the surface film is 25-30 A thick, the expected width of a monolayer containing phospholipid, free cholesterol and protein. Lipoprotein lipase reduced the chylomicron-triglyceride core without disrupting the surface film.

Metabolism of chylomicrons in vivo: Lipolysis of chylomicrons in vivo results in smaller particles, called "remnants", which contain the same amount of cholesterol ester but less triglyceride. These particles are subsequently removed from the blood by liver. Studies were undertaken to determine the effect of hydrolysis within the capillary bed of perfused tissue on the structure of chylomicrons and to compare the resultant particles with those produced by incubating chylomicrons with lipoprotein lipase. The venous blood from lactating rat mammary tissue perfused 2-3 min with chylomicrons contained lipid-depleted particles similar in structure to those produced in vitro. Studies are now in progress to characterize and to compare these venous blood remnants with other lipoproteins in blood.

Transport of chylomicron-fatty acids across mammary capillary endothelium: Capillaries develop in mammary tissue, along with the alveolar sacs, in response to the stimulus of pregnancy, and regress when lactation is terminated. Morphological studies show clear differences between developing, mature and regressing endothelial cells. Lipoprotein lipase activity in mammary tissue, which is low during the first 20 days of pregnancy, increases 2-3 days before parturition and remains high throughout lactation. The presence of lipoprotein lipase activity within endothelial cells may serve as a marker of endothelial cell maturity.

The capillaries in lactating mammary gland consist of a continuous endothelium surrounded by a continuous basement membrane. The endothelial cells contain numerous microvesicles and vacuoles and have luminal projections which seem to entrap chylomicron particles. The relationship between the endothelial projections, lipoprotein lipase and chylomicrons is not known. Entrapment of chylomicrons could be increased by conditions which favor uptake of triglyceride. Morphological studies show that the plasma membrane of the endothelial projections may contain more free cholesterol than intracytoplasmic membranes.

Role of the capillary endothelium in the uptake of FFA: Adipose tissue was perfused with blood containing a high concentration of albumin-bound FFA and studied with an electron microscopic cytochemical technique to determine the mode of transport of FFA across the capillary wall. The reaction product, Pb-fatty acid precipitates, was found in microvesicles indicating vesicular transport of FFA across the endothelium. Reaction product was also found in interendothelial clefts. However, the tight junction at the luminal endothelial border was intact, suggesting that FFA may have entered the intercellular sites via microvesicles.

Significance to Biomedical Research in the Program of the Institute: FFA mobilized from adipose tissue provide a major part of the energy expended by the body during fasting. The FFA are transported to the tissues in blood bound to plasma albumin. The nature of transport of FFA across the capillary wall, during both mobilization and uptake (by muscle, heart and other tissues), has not been studied. Our studies in perfused adipose tissue show that capillary endothelial cells can remove FFA from plasma and transport them across the endothelium within microvesicles. The studies in chylomicrons incubated with lipoprotein lipase indicate that albumin has a specific role in the transfer of FFA across the surface film of chylomicrons. These findings are compatible with the hypothesis that albumin may bind to the polar end of FFA when it removes FFA from chylomicrons or from the luminal surface of capillaries of adipose tissue during FFA mobilization, and that the nonpolar end of FFA may bind to the endothelial plasma membrane for transport across the endothelium, within microvesicles, during both mobilization and uptake.

Alterations in the metabolism of plasma triglyceride occur in various hyperlipemic states. In some, synthesis and secretion of lipoproteins are abnormal whereas in others, uptake is reduced, with or without changes in lipoprotein lipase activity. Localization of lipoprotein lipase synthesis and activity in tissues and study of the nature of the reaction of lipoprotein lipase with chylomicrons and other lipoproteins could provide valuable information concerning factors that regulate and affect clearance of triglyceride from blood.

Proposed Course: Our cytochemical findings show that hydrolysis of chylomicron-triglyceride by lipoprotein lipase occurs within the capillary endothelium of adipose tissue. Although fat cells are thought to be the main source of this enzyme the manner in which the enzyme could be transferred to the endothelium has not been determined. An attempt will be made to develop an immuno-cytochemical technique for localizing lipoprotein lipase. With such a technique, one could determine whether the enzyme is present in parenchymal cells, pericytes, or in connective tissue components of the tissue. The effect of hormones and other factors on transport of the enzyme from sites of synthesis to sites of action could also be studied.

Sites of lipoprotein lipase synthesis and activity will be studied also in perfused lactating rat mammary tissue, skeletal muscle, and heart. Uptake and hydrolysis of triglyceride by arteries in different tissues and the effect of diabetes and insulin on these processes will be studied.

The nature of the enzyme-substrate complex formed between lipoprotein lipase and chylomicrons will be studied *in vitro* and *in vivo*. It is believed that the small amount of protein present on the chylomicron surface is necessary for the formation of the complex. Chylomicrons reacted with purified lipoprotein lipase will be studied structurally to determine if the enzyme has a direct effect on the chylomicron surface coat. The nature of the interaction *in vivo* between chylomicrons and lipoprotein lipase, presumably located on the luminal surface of capillary endothelial cells, will be studied, using a combination of immuno-cytochemical, freeze-etch and other techniques.

Hypertriglyceridemia and fatty liver occur in uncontrolled diabetes and in late pregnancy. The circulating lipoproteins in these states will be isolated and studied with the electron microscope to compare their structure with that in control animals. Since intestinal epithelial cells and hepatic parenchymal cells both synthesize plasma lipoproteins, and liver cells can store triglyceride, various components of these cells will be isolated for studies of nascent lipoproteins and cellular lipids in control, pregnant and diabetic animals.

Honors and Awards: None

Publications:

Blanchette-Mackie, E.J. and Scow, R.O. Effects of lipoprotein lipase on the structure of chylomicrons. *J. Cell Biol*. In press.

Serial No. NIAMDD-LNE-7
1. Nutrition and Endocrinology
2. Membrane Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mechanism of Action of Hormones on Adenylate Cyclase Systems

Previous Serial Number: NIAMD-LNE-7

Principal Investigator: Dr. Martin Rodbell

Other Investigators: Dr. Michael C. Lin, Visiting Scientist
Dr. Yoram Salomon, Visiting Fellow
Dr. James P. Harwood, Guest Worker
Dr. Boanerges Rubalcava, Guest Worker
Dr. Constantine D. Londos, Guest Worker
Ms. Vivian Goldberg, Predoctoral Fellow

Cooperating Units: Dr. Victor Hruby, University of Arizona

Man Years:

Total: 4
Professional: 3
Other: 1

Project Description:

Objectives: To determine the mechanism of action of various hormones on adenylate cyclase systems in membranes from various animal cells.

Methods Employed: Cell membranes from a variety of cell types are purified by differential centrifugation. Adenylate cyclase activity and binding of labeled hormones were carried out by procedures developed in this laboratory. Various types of chromatographic procedures, solid-phase synthesis of peptide hormones, and methods for measuring incorporation and binding of nucleotides were also employed.

Major Findings:

I. Regulation of Adenylate Cyclase Systems

A. Mechanism of Action of Glucagon on Adenylate Cyclase in Hepatic Membranes

The actions of a variety of hormones are exerted through two interdependent regulatory enzyme systems, the adenylate cyclase system which produces cyclic AMP in response to hormones and the cyclic AMP-dependent protein kinase system. The adenylate cyclase system is constructed within the matrix of the plasma membrane of the target cell and contains specific receptors for each hormone. The receptors appear to be separate proteins from the enzyme that catalyses the formation of cyclic AMP from ATP. Although the overall structural features of the enzyme system remain unknown, several lines of evidence suggest that the transduction process responsible for amplification and regulation of hormone action occurs through an energy transaction at the boundary interface between receptor and enzyme subunits.

In the case of the glucagon-sensitive adenylate cyclase system in hepatic plasma membranes, the energy transaction appears to involve the concerted interaction of GTP (or ATP) and of the NH_2-terminal histidine residue of glucagon with a transition state metal ion. Acidic phospholipids are required for this concerted interaction and may participate either by maintaining the structural orientation of receptor and enzyme subunits or by functionally interacting, through their acidic head groups, with the metal ion involved in the transduction process. Sulfhydryl groups are also critically involved in transduction and may be liganded to the metal ion in such a fashion that interaction of the glucagon histidine residue and of GTP cause perturbations of the boundary interface and release of the sulfhydryl groups.

Kinetic studies of glucagon binding and activation of adenylate cyclase have revealed that glucagon causes a time- and concentration-dependent change in the enzyme system. The binding process displays positive cooperativity (Hill coefficient, 1.5) which can account for the slow reversibility of hormone binding as a function of time and hormone concentration. It is presumed that cooperativity reflects subunit interactions; the NH_2-terminal histidine residue is required for cooperative binding. The glucagon-induced change in the enzyme system is a rate determining step for the interaction of GTP with the regulatory (or transduction) site; the affinity of this site for GTP is increased as a function of either glucagon concentration or of the time necessary to produce hormone occupied states that are compatible for nucleotide interaction. In the presence of maximal concentrations of GTP, minimal occupation of the glucagon receptor is required for near-maximal activation of the enzyme system. In contrast, at minimal nucleotide concentrations, high receptor occupancy is required to achieve the same levels of activation of the enzyme. This reciprocal relationship is explained by the change in nucleotide affinity induced by glucagon action.

GTP interaction with the enzyme system results not only in activation of the enzyme but also in markedly enhanced rates of glucagon dissociation from the receptor. The GTP-induced change in rate of dissociation is not accompanied by a change in the rate of association of glucagon with the receptor; the net result is a lower steady state level of hormone-occupied states when the rate of glucagon association, which is concentration dependent, is less than the rate of dissociation. Thus, whereas glucagon binding is a rate-determining step in nucleotide interaction, nucleotide interaction results in release of the hormone. This type of "ping-pong" relationship explains the rapid reversibility of the activated states of adenylate cyclase when the hormone concentration is reduced. Moment-to-moment control of cyclic AMP production is best seen with minimal occupation of the receptor and maximal concentrations of GTP (or ATP) which has a 1000-fold lower affinity for the regulatory or transducer site.

Based on the above observations, a new theory of hormone action, termed "concerted" is proposed and is described by the following series of events

$$\begin{tikzcd}
H + E & EH_1 \arrow[r, shift left] \arrow[d, shift right, "N"'] & EH_j \arrow[l, shift left] \arrow[d, shift right, "N"'] & \text{(inactive states)} \\
& NEH_1 \arrow[u, shift right] \arrow[r, shift left] \arrow[ul, bend left] & NEH_j \arrow[l, shift left] \arrow[u, shift right] & \text{(active states)}
\end{tikzcd}$$

in which the hormone (H) interacts with the enzyme system (E) to produce a series of inactive, hormone occupied states (EH_{1-j}) each of which has a different affinity for the nucleotide (N) as a function of the number of receptors occupied. Occupation of the nucleotide site results in activation of the enzyme and in a change in the state of the enzyme that results in release of hormone from its receptor. The ligand-induced changes in the enzyme system can be either of the "concerted" or "sequentially-induced" type. It is also possible that the changes are "stochastic" rather than "deterministic" in character.

B. Adrenal Cell ACTH Sensitive Adenylate Cyclase System: ACTH stimulates the activity of adenylate cyclase in purified plasma membranes from adrenal cortical cells (bovine or rat). As observed with the glucagon-sensitive system in liver membranes, GTP (10^{-8} to 10^{-6} M) is required for activation of the adrenal system in the presence of ACTH. Metal ion chelators, such as EGTA or EDTA mimick the effects of ACTH and also require GTP for activation. The chelators act at very low concentrations (10^{-10} to 10^{-8} M). In the presence of maximal concentrations of the chelators, addition of ACTH does not cause further activation of the enzyme. Although studies in other laboratories have suggested that calcium ion is involved in ACTH action, our studies suggest that calcium ion plays only an inhibitory role; extensive treatment of the membranes with calcium-chelating reagents fail to diminish the actions of ACTH on adenylate cyclase. It appears that a metal ion, probably of the transition state type, is involved in the concerted actions of ACTH and nucleotides, as observed with the glucagon sensitive system.

C. The Multi-receptor Fat Cell Adenylate Cyclase System:

1. Effects of guanyl nucleotides: In fat cells of the rat, adenylate cyclase is stimulated by ACTH, glucagon, secretin, epinephrine, and TSH. These hormones activate the enzyme through distinct receptors. Basal activity (unstimulated) is very high in either fat cell "ghosts" or in highly purified plasma membrane preparations from these cells.

A new mechanism of regulatory control of adenylate cyclase has been discovered recently with the fat cell system. GTP (10^{-7} to 10^{-5} M) causes marked inhibition of basal activity. Inhibition is caused by phosphorylation of the enzyme through a phosphotransferase in the plasma membrane which uses GTP preferentially (ITP is much less effective; no other nucleotide affects the system) as the phosphate donor. When GTP is exhausted, restoration of normal activities occurs suggesting that a phosphatase in the membranes causes de-phosphorylation of the enzyme. The properties of the GTP transferase enzyme and the phosphatase remain unknown.

As was observed with the glucagon- and ACTH-sensitive adenylate cyclase system, GTP also enhances the reactivity of the fat cell system to glucagon, secretin, ACTH, epinephrine, and TSH, which suggests that the same type of concerted mechanism for hormone and nucleotide action exists in the multi-receptor fat cell system.

The complex nature of the fat cell system raises the possibility that the enzyme including phosphotransferase, phosphatase, receptors, and adenylate cyclase, consist of a large aggregate of enzymes and regulatory components constructed within the matrix of the plasma membrane.

2. Effects of fluoride ion: Fluoride ion stimulates the activity of all adenylate cyclase systems. The mechanism of fluoride activation remains unknown. Divalent cations are required and the kinetic characteristics with respect to substrate utilization are the same as observed with hormonal activation. Although previous studies have suggested that fluoride ion activates the enzyme through a process completely unrelated to hormonal activation, recent studies with the fat cell system suggests that fluoride ion may affect the same transduction process affected by nucleotides and hormones. Fluoride activation is temperature dependent in contrast to hormonal activation of the fat cell system. It was discovered that addition of fluoride ion with the hormones at incubation temperatures at which only hormones activate the system results in abolishment of hormonal activation. Fluoride abolishes hormone action by a non-competitive process and inhibits the actions of all hormones acting on the system. Based on these findings, it appears that fluoride acts at a site related to the transduction process. Since fluoride inhibits the effects of chelators and nucleotides on the adrenal and hepatic system, it is possible that fluoride, in its selective inhibition of hormone action, alters the ability of nucleotides to interact with the transduction site. In the fat cell system, increasing the temperature allows fluoride ion to activate the enzyme system possibly through the same transduction site which no longer operates with hormones and nucleotides when occupied by fluoride ion.

II. GTP-protein Kinase

Cyclic AMP-dependent protein kinase(s) catalyse the transfer of the terminal phosphate of ATP to a variety of regulatory enzymes including those involved in glycogen and fat metabolism. Cyclic AMP also regulates a number of processes involved in protein synthesis, ion transport, gene expression, secretion of hormones and enzymes. The role of cyclic AMP-dependent protein kinase in these processes is poorly defined. We have discovered recently a new regulatory enzyme which may be involved in the regulation of some of these processes. It was found that the terminal phosphate of GTP is transferred to histones by an enzyme in fat cells, hepatic cells, and muscle. The activity of the enzyme is very low in the absence of ATP and cyclic AMP. Addition of these nucleotides results in marked stimulation of GTP protein kinase activity. Activation by ATP and cyclic AMP is due to conversion of the unactivated form of cyclic AMP-dependent protein kinase to its active form. The active enzyme then phosphorylates GTP protein kinase. Thus, cyclic AMP dependent protein kinase is fundamental for the activity of GTP protein kinase. GTP protein kinase has been separated from cyclic AMP-dependent protein kinase and from phosphorylase b kinase. GTP-kinase is not involved in glycogen or fat metabolism.

Significance to Bio-medical Research and the Program of the Institute: Adenylate cyclase is a key regulatory enzyme system for the initial actions of a number of peptide hormones, biogenic amines, and prostaglandins. A new theory of hormone action, based on our studies of the glucagon-sensitive system in hepatic cells, has been proposed which brings into focus a number of aspects of hormone action hitherto unrecognized. The theory explains the kinetics of hormone action and binding at the receptor. Most importantly, it shows that activation of adenylate cyclase is a two-step process requiring hormone interaction with receptor in order to increase the binding of nucleotides at a crucial step, termed transduction, in the activation of the enzyme. The theory explains also the finely tuned control of cellular metabolism and shows how, under conditions of minimal nucleotide concentration, the cell may appear to be resistant to hormone action. Although intimate details of the theory remain to be investigated, thus far the general aspects of the theory appear to apply to a number of hormone-sensitive adenylate cyclase systems. As a model for hormone action in general, the "concerted" theory of hormone and nucleotide action on adenylate cyclase system may also contain principles that apply to hormones such as insulin, growth hormone, prolactin which also interact with cell membranes but apparently not through the adenylate cyclase system.

The discovery of a new regulatory enzyme, GTP-protein kinase, that is activated by cyclic AMP-dependent protein kinase may help to resolve some of the questions concerning how cyclic AMP regulates cellular metabolism and structure.

Proposed Course: Continued efforts will be made to isolate and characterize the various components of adenylate cyclase systems in liver, adipose, and adrenal cells. Increased emphasis will be placed on the kinetics of nucleotide binding and action on these systems as a function of hormones and chelators. Purification of GTP-protein kinase from hepatic and fat cells and attempts to discover the substrates of the enzyme in these cells will be made.

Honors and Awards: Awarded Jacobeaus Prize by Scandanavian Endocrine Society.

Publications:

Rodbell, M. Methods for the Isolation of Rat Liver Plasma Membranes and Fat Cell "Ghosts"; An Assay Method for Adenylate Cyclase. In: Chasin, M. (Editor) Methods in Cyclic Nucleotide Research. Vol. 3. Marcel Dekker, Inc., (New York), 1972, p. 101-124.

Rodbell, M. Cell Surface Receptor Sites. In: Anfinsen, C.B., Goldberger, R.F. and Schechter, A.N. (Editors) Current Topics in Biochemistry. Academic Press (New York), 1972, p. 187-218.

Rodbell, M. Regulation of Glucagon Action at Its Receptor. In: Lefebvre, P.J. and Unger, R.H. (Editors) Glucagon. Pergamon Press (New York), 1972, p. 61-75.

Rodbell, M. and Salomon, Y. Activation of a GTP-Specific Protein Kinase by Adenosine 3'5' Monophosphate-Dependent Protein Kinase in Fat Cells of the Rat. J. Biol. Chem. 248 (1973).

Rubalcava, B. and Rodbell, M. The Role of Acidic Phospholipids in Glucagon Action on Rat Liver Adenylate Cyclase. J. Biol. Chem. 248 (1973).

Serial No. NIAMDD-LNE-8
1. Nutrition and Endocrinology
2. Nutritional Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biochemical Studies Related to Nutrition

Previous Serial Number: NIAMDD-LNE-8

Principal Investigators: Drs. J. G. Bieri, J. N. Williams, Jr., and H. G. Windmueller

Other Investigators: Dr. R. E. Poukka Evarts, S. L. Thorp and A. E. Spaeth

Cooperating Units:
Dr. D. P. Tschudy, MD, NCI
Dr. J. B. Levine, MD, NCI
Dr. W. T. London, DVM, NINDS
Dr. P. Farrell, MD, NIAMDD

Man Years
Total: 7
Professional: 5
Other: 2

Project Description:

Objectives - To determine the nutritional, biochemical and physiological role of essential nutrients for experimental animals. To define the metabolic function of certain nutrients and to study nutritional effects on the tissue levels of various metabolites.

Methods Employed - Rats, hamsters and guinea pigs are fed specially prepared, highly purified diets that contain adequate amounts of each nutrient known to be required by the particular species. The effects of specific deficiencies and imbalances are assessed by measurement of physiological, chemical and enzymological changes in the animal, its tissues and excreta.

The relationship of nutrition to other scientific areas, such as neurology and pathology, is studied.

Major Findings

A. Metabolism and Function of Fat-Soluble Vitamins (J. G. Bieri and R. Poukka Evarts)

In continuing studies of the metabolism of various forms of vitamin E, γ- and α-tocopherols were fed simultaneously to vitamin E depleted rats in a proportion of 2:1, respectively (a ratio found in many U.S. diets). Analyses after two weeks revealed that the ratio of γ-:α-tocopherol varied widely in different tissues, from 0.14 in liver to 0.35 in adipose tissue. In other experiments, corn oil or soybean oil (γ-tocopherol:α-tocopherol ratios of 3:1 and 6:1, respectively) were fed to rats for 12 weeks. Wide variations in tissue ratios of γ-:α-tocopherol were again found, with liver the lowest at 0.14 while heart, 0.50 and adipose tissue, 0.74 were highest. In concomitant studies, the relative vitamin E activities of α- and γ-tocopherols were compared in assays with rats, chicks and hamsters. γ-tocopherol had activity ranging from 5 to 12 per cent (average of 9 per cent), of the activity of α-tocopherol. These studies indicate that γ-tocopherol has significant biological activity and can make an appreciable contribution to the total vitamin E activity of human diets.

In collaborative studies with Dr. W. T. London (NINDS) on the protein and calorie requirements of pregnant rhesus monkeys purified diets were devised which were consumed essentially quantitatively. Varying combinations of protein and energy were fed for the last four-fifths of pregnancy. Per kilogram of body weight, 2.1 grams of protein (casein) and 88 kilocalories permitted normal maternal weight gain and positive nitrogen balance. The infant monkeys appeared normal and had body weights in the normal range.

It has been reported that the vitamin E status of rats affects several liver enzymes involved in heme synthesis. In a comparison of livers from normal and vitamin E deficient rats, no differences were found in the activities of delta-aminolevulinic acid (ALA) synthetase or in ALA dehydrase (in collaboration with Drs. Tschudy and Levine, NCI).

B. Net Uptake and Metabolism of Plasma Glutamine by Intestine (H. G. Windmueller)

It is generally believed that circulating glucose constitutes the major metabolic fuel for the intestine. We have now shown, however, with a preparation of isolated, blood-perfused rat intestine developed recently in this laboratory, that in addition to glucose, there is a large net uptake of glutamine from the circulation (ca. 30% clearance in each pass through the gut vasculature). There is no net uptake of any other amino acid. Perfusion studies with L-methionine-DL-sulfoximine, an inhibitor of glutamine synthesis, showed that glutamine can be produced by the preparation but at a rate lower than the rate of utilization. On a molar basis, net uptake of plasma glutamine by the gut totals 65% the uptake of plasma glucose. Arteriovenous concentration measurements across intestine showed that rates of plasma glutamine uptake comparable to those found in the isolated gut preparation occur *in vivo* in fasted dogs, cats, hamsters, monkeys and in conventional and germfree rats, thus indicating that the phenomenon is widespread among animals and occurs independent of the gut microflora. No net uptake of plasma glutamine was found across any major organ vascular bed of the rat except across the gut. In the rat (germfree) glutamine nitrogen taken up by the gut can be accounted for by citrulline (34%), alanine (33%), ammonia (23%) and proline (10%) released by gut back into the blood. When [U-^{14}C] glutamine was added to the recycled blood perfusing isolated intestine, ^{14}C was recovered in respired CO_2 (35%); in perfusate citrulline (5%), glutamic acid (3%), proline (3%), alanine (1%), citric acid (6%), lactic acid (3%) and other organic acids (9%); and in tissue protein (11%). Further studies in vivo in which rat intestine was exposed to vascular [U-^{14}C] glutamine for <1 min. revealed that gut metabolism of plasma glutamine occurs largely in cells of the small intestinal mucosa and apparently proceeds via glutamic acid. From other studies it was shown that a large net uptake of plasma glutamine continues even when the gut is transporting massive quantities of glutamic acid from the gut lumen into blood. Tissue analyses in fasted rats showed that, compared with other organs, the rat intestinal mucosa has a very low glutamine concentration (0.2 μmoles/g), about 5% the concentration in liver and skeletal muscle, but not unusual levels of glutamic acid or asparagine. Further preliminary studies indicate that the gut mucosa may require exogenous glutamine to support normal rates of protein synthesis. Enzymatic hydrolysis, with a highly purified asparaginase from E. coli, of all asparagine and glutamine of blood used for perfusing isolated rat gut preparations resulted in a 75% reduction in the incorporation of perfusate L-[^{3}H] lysine into mucosal protein, while incorporation into protein of other regions of the preparation (small intestinal muscularis, cecum, color, mesentery) was only slightly inhibited. It seems likely that this 75% inhibition was due to a perfusate glutamine deficiency since normal animal cells are thought not to require exogenous asparagine.

Biochemical Studies of Cytochromes (J. N. Williams, Jr.)

Studies are continuing on a sub-population of liver particles obtained by high speed centrifugation of a mitochondrial fraction after the bulk of the heavy mitochondria are spun down at lower speeds. On sucrose density gradients the particles peak at a significantly lower density than other mitochondrial fractions, but at a significantly higher density than microsomes. A series of enzymes located in different parts of the mitochondrion have been studied in these particles. The specific activities of cytochrome oxidase (an inner membrane marker) and monamine oxidase (an outer membrane marker) are the same as in the more dense mitochondrial subfractions. The specific activity of adenylate kinase (an enzyme believed to be located between the outer membrane and the inner membrane) falls markedly in the less dense particles while malate dehydrogenase (an inner matrix enzyme) shows the same specific activity in all of the subfractions. The specific concentration of cytochrome c, an easily extractable protein, is more concentrated in the less dense particles. Respiratory control and NADH oxidase, which represent a measure of integrity of the inner and outer membranes of mitochondria, respectively, are similar in the various subfractions. It is of interest, however, that G-6-Pase activity (a microsomal marker) correlates almost 1:1 with cytochrome oxidase in the various subfractions on sucrose density gradients. Whether this is fortuitous or an indication of a combination of mitochondria still attached to the endoplasmic reticulum via the outer mitochondrial membrane cannot be answered until electron micrographs of the particles can be obtained.

Preliminary studies of hormonal effects on mitochondrial components have been initiated. Earlier work by others have indicated that mitochondrial enzymes and other components are quite refractory to hormonal manipulations in the rat. Our interest has focused on the soluble mitochondrial component, cytochrome c. Injections of cortisol or testosterone into rats fed normal, low or high protein diets have produced little or no significant effects on cytochrome c concentrations of liver or heart. Injections of allylisopropyl acetamide (AIA), an inducer of δ-aminolevulinic acid synthetase, tended to produce a slight rise in liver cytochrome c concentrations after 0.5-3 hrs. with a decrease thereafter. Glucagon, a hormone producing a dramatic increase in mitochondrial histidine-pyruvate aminotransferase (thought to be synthesized on the microsomes and then transferred to the mitochondrion similarly to cytochrome c) was tested for its effects on cytochrome c concentrations both in the cytosol plus microsomes and mitochondria. Given alone by injection there was no effect on mitochondrial cytochrome c, but there was an increase in cytosol plus microsomal cytochrome c. When glucagon plus AIA were injected into rats, the specific concentrations of the mitochondrial heme proteins cytochrome oxidase, cytochrome b, and cytochrome c were increased about 1.5-1.8 fold. The concentration of cytochrome c in the cytosol plus microsomes was increased by about the same amount. Work is continuing on the influence of hormones on mitochondrial proteins.

D. Significance to Bio-Medical Research and the Program of the Institute

A more complete understanding of the nutrition, biochemistry and metabolism of essential amino acids, proteins, vitamins, minerals and fatty acids in different living organisms can be expected to contribute still further to our knowledge of the roles of these essential nutrients in human beings. It is well established that the nutrition of man plays a role in the etiology of many degenerative and metabolic diseases, certain infectious and neurological diseases and some types of cancer. Basic studies in nutrition and biochemistry of nutrients may provide the means to prevent or cure some of these diseases.

E. Proposed Course of Project

Clarification of nutritional responses and explanation of interrelationships at the biochemical level remain the primary goals of the project.

F. Publications

LaRosa, J. C., Levy, R. I., Windmueller, H. G. and Fredrickson, D. S.: A comparison of the triglyceride lipase of liver, adipose tissue and post-heparin plasma. J. Lipid Res. 13: 356-363, 1972.

Peake, I. R., Windmueller, H. G., and Bieri, J. G.: A comparison of the intestinal absorption, lymph and plasma transport, and tissue uptake of α- and γ-tocopherols in the rat. Biochim. Biophys. Acta 260: 679-688, 1972

Reid, M. E. and Bieri, J. G.: Nutrient requirements of the guinea pig. In "Nutrient Requirements of Laboratory Animals" 2nd ed., pp 9-14, National Acad. of Sciences, 1972

Bieri, J. G. and Evarts, R. H. Poukka: Vitamin E Nutrition in the Rhesus Monkey (36632). *Proc. Soc. for Exp. Biol. and Med*. 140: 1162-1165, 1972

Bieri, J. G.: Kinetics of tissue α-Tocopherol depletion and repletion. *Ann. N.Y. Acad. Sci*. 203: 181-191, 1972.

Bieri, J. G. and R. Poukka Evarts: Tocopherols and fatty acids in American diets. *J. Am. Dietetic Asso*. 62: 147-151, 1973.

Windmueller, H. G., Herbert, P. N., and Levy, R. I.: Biosynthesis of lymph and plasma lipoprotein apoproteins by isolated perfused rat liver and intestine. *J. Lipid Res*. 14: 215-223, 1973.

Krauss, R. M., Windmueller, H. G., Levy, R. I. and Fredrickson, D. S.: Selective measurement of two different triglyceride lipase activities in rat post-heparin plasma. *J. Lipid Res*.: In press.

Eisenberg, S., Windmueller, H. G., and Levy, R. I.: The metabolic fate of rat and human lipoprotein apoproteins in the rat. *J. Lipid Res*.: In press

Serial No. NIAMDD-LNE-9
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Structure and function of barnase and barstar, an extracellular ribonuclease (barnase) and its intracellular inhibitor (barstar) from Bacillus amyloliquefaciens.

Previous Serial Number: SAME

Principal Investigator: Dr. Robert W. Hartley

Other Investigators: None

Cooperating Units: Large-scale laboratory, Office of Chief, LNE, NIAMDD. Dr. Robert W. Hartley (on leave without pay, July-August) Wellcome Fellow at Sub-division of Protein Chemistry, Medical Research Council, Laboratory of Molecular Biology, collaboration with B.S. Hartley, J. Sperling, D. M. Blow and R. C. Sheppard, Cambridge, England.

Man Years

Total:	1.5
Professional:	1.0
Other:	0.5

Project Desscription:

Objectives: To further purify, characterize and investigate this enzyme and its natural inhibitor by physical, chemical, and biological techniques. To develop methods of culture, genetic manipulation and genetic analysis so that the organism and its product enzyme-inhibitor pair may be applied to a broad structure, function and synthesis.

Methods Employed: B. amyloliquefaciens (strain H and derivatives and several other strains all of which were formerly considered to be strains of B. subtilis) are grown in synthetic media in a variety of batch and continuous flow fermentations. Isolation of barnase involves: 1) adsorption onto P-cellulose from acidified culture medium, followed by stepwise elution, 2) desalting by Sephadex chromatograpy, 3) gradient chromatography on CM-cellulose. The inhibitor (Barstar) is extracted from the previously acidified cells at pH 8.5. It is purified by acid precipitation, DEAE-cellulose, and Sephadex G-75 chromatography and by making use of its specific binding to purified enzyme.

Work on the determination of amino acid sequence involves amino acid analysis, enzymatic digestions, peptide separations, and peptide sequencing by the combined Edman-dansyl techniques. Amino acid analysis is by automated liquid chromatography using a JEOLCO analyzer. Dansyl amino acid derivatives are identified by thin layer chromatography on polyamide layers. Peptide separations are obtained by Sephadex and ion-exchange chromatography.

The reversible transitions undergone by barnase and barstar and their derivatives under the influence of temperature changes or denaturing agents is studied primarily by ultraviolet and fluorescence spectroscopy, but also by optical rotatory dispersion, exclusion chromatography, and with the use of proteolytic enzymes. These techniques are also used to study complementation reactions between natural and synthetic peptide sequences of the two proteins as well as conformational properties of such peptides alone. Studies of the peptide derivatives also include use of antibodies, chromatography, crystal growth, etc.

Equilibrium ultracentrifugation has been used to measure the molecular weights of the enzyme, the inhibitor, and their complex, and to determine the molecular weight homogeneity of each. Disc and equilibrium pH gradient electrophoresis have also been used to assess homogeneity and to demonstrate the reversibility of the complex formation. Bacterial mutants are obtained by chemical mutagenesis of vegetative cells and by gamma irradiation of spores. A variety of techniques are used to isolate ribonuclease, protease, amylase, nutritional and antibiotic resistant mutants.

Radioactive amino acids are incorporated into barnase and into nascent barnase peptides by their addition to appropriately prepared cells.

Crystals of barnase are grown in hanging drops (10 ul) suspended over suitable solvents. Isomorphous crystals containing heavy atoms are prepared by soaking crystals in solutions of appropriate heavy atom compounds. The three dimensional structure of barnase is being determined by methods of X-ray crystallography.

The Merrifield procedure is being used to synthesize portions of the barnase sequence.

Major Findings: A 4.5 Å solution of the barnase structure has been obtained by studies of a single crystal, using the special three-fold symmetry of the barnase crystal to obtain phase information. It is not possible to trace the peptide chains at this resolution, nor does it appear that further resolution will be possible with this technique. However, two good isomorphous replacements have been obtained, using gold cyanide and mercury salycilate. Full data at 2.5 Å resolution has been taken for these two as well as for the "native" crystal and a solution at that resolution is anticipated.

A peptide complementation system for barnase has been discovered. Barnase (1-103) missing eight residues from its C-terminal, binds the peptide (88-100) with an association constant in theorder of 10-5 to 10-6 and with restoration of at least 50% of its ribonuclease activity. The peptide (99-108) also complements, but requires a thousand-fold excess. No complementation is obtained with the missing octapeptide (103-110) itself.

The reaction between barnase and the detergent sodium dodecyl sulfate can be represented by a simple model involving a reversible, all-or-none conformational transition from which the number of detergent molecules bound to each form can be determined. A reversible transition produced by the cationic detergent cetyl-trimethylammonium bromide appears to be somewhat more complex and produces entirely different spectral changes.

Changes in the bacterial growth medium, introduced for engineering reasons, have allowed the production of a substantial quantity (20-30 mg) of barnase A. This is an active, but chromatographically different, form of barnase which is probably a precursor of the barnase normally isolated.

Significance to Bio-Medical Research and the Program of the Institute: The small size, stability, easily measured enzyme activity and absence of disulfide bridges in barnase made it an excellent choice for a long-range study of the relation between the sequence of an enzyme and its structure and function. The inhibitor is an even simpler protein and should be useful in a similar fashion. Together, they offer a model system for the study of a protein-protein interaction.

The nature and extent of the thermal transition of barnase gives strong support to the concept that the three-dimensional structure of a protein may be completely determined by its amino acid sequence. The simplicity and all-or-none nature of the transition should make it useful for thermo-dynamic analysis of the factors involved in maintaining a folded protein structure.

The production of both of these proteins by the same organism is also of biological interest.

Proposed Course of Project: X-ray structure work on barnase crystals will continue, along with efforts to obtain usable crystals of barstar and the barnase-barstar complex. Sequencing of barstar will continue.

Attempts will be made to determine the difference between barnase and its presumed precursor, barnase-A.

Experiments to further characterize the reactions between barnase (1-103) and its complementing peptides will be carried on, along with a search for other complementing systems.

The nature of protein detergent interactions will be further investigated using barnase plus sodium dodecyl sulfate or cetyl trimethylammonium bromide as model systems.

Honors and Awards: None

Publications:

Hartley, R. W. and Rogerson, D. L. Jr.: Production and purification of the extracellular ribonuclease of *Bacillus amyloliquefaciens* (Barnase) and its intracellular inhibitor (Barstar). I. BARNASE. *Preparative Biochemistry* 2: 229-242, 1972.

Hartley, R. W., Rogerson, D. L. Jr., and Smeaton, J.R.: Production and purification of the extracellular ribonuclease of *Bacillus amyloliquefaciens* (Barnase) and its intracellular inhibitor (Barstar). II. BARSTAR. *Preparative Biochemistry* 2: 243-250, 1972

Hartley, R. W. and Smeaton, J. R.: On the reaction between the extracellular ribonuclease on *Bacillus amyloliquefaciens*. *J. Biol. Chem.* (in press).

Serial No. NIAMDD-LNE-10
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on structure and function of nucleic acids.

PRevious Serial Number: SAME

Principal Investigator: Dr. G. W. Rushizky

Other Investigators: Mr. J. E. Mozejko, Mr. D. L. Rogerson, Jr., and Dr. H. A. Sober

Cooperating Units: Large-scale Laboratory, LNE, NIAMDD

Man Years

Total:	2.3
Professional:	2.3
Other:	0

Project Description:

Objectives: To study the enzymology and chemistry of nucleic acids and to develop new methods for the determination of nucleotide sequences in RNA and DNA.

Methods Employed: Various procedures for the preparation and fractionation of large oligonucleotides derived from RNA (MS2 virus) and DNA (fd virus) were investigated.

1) Charged groups were incorporated into polyacrylamide gels and electrophoresis was employed to provide an increased capacity and/or additional parameters for the fractionation of tRNA-size oligonucleotides. Substituents used were acrylic acid, CM-cellulose (Na-salt), acid-solubilized DEAE-cellulose and polyethyleneimine; as well as combinations of the above.

2) fd virus preparations contain up to 25% of "light" fd particles, but with 25% less DNA than fd. These light particles resemble fd itself by various tests but are non-infectious (or highly reduced in biological activity). DNA from both particles was compared by examination of partial hydrolysates prepared with micrococcal nuclease.

3) Native viruses, MS2 or fd, were pre-treated with detergents or reagents that cleave peptide chains and then exposed to ribonucleases or DNases. After phenol treatment to remove enzyme and viral protein, the DNA and RNA of the viruses was examined (by sucrose sedimentation, gel electrophoresis and chromatography on paper or DEAE-cellulose) for the extent of RNA or DNA hydrolysis.

Major Findings: 1) Gel electrophoresis (GE) with positive or negative substituents alone resulted in gels subject to "creeping",i.e. disruption of gels and movement out of tube upon prolonged electrophoresis. A decrease in the amounts of substitutents incorporated into the gels decreased creeping but also lowered their capacity for additional oligonucleotides to a point close to the capacity of unsubstituted gels. Among "balanced" gels, combinations of acrylic acid and polyethyleneimine gave the best results with respect to improved resolution (increase in total numbers of oligomer bands over that obtained with unsubstituted gels). However, while resolution was improved, capacity, the primary objective, was not. 2) fd DNA from light fd particles moves like fd DNA from mature virus upon sucrose gradient or CsCl sedimentation. Partial hydrolysates with micrococcal nuclease and GE at various gel concentrations (6-18%) shows similar patterns of oligonucleotides. Differences between the DNAs, if any, are not obvious enough to be detected under the conditions employed. This study was discontinued. 3) While both intact MS2 and fd virus are resistant to RNases or DNases, respectively, modification of the protein coat with SDS or succinic anhydride, N-bromosuccinimide (NBS), or Clelands reagents (dithioerythritol or dithiothreitol) provides viral derivatives that allow in situ enzymatic hydrolysis of the nucleic acids. With MS2, RNase T_1 and Barnase gave patterns of large oligonucleotides by GE at 6 and 12% gel concentration. Unexpectedly, the same viral derivatives of MS2 provided no protection against RNase T_2 or pancreatic RNase, while micrococcal nuclease was not able to attack the modified MS2. With RNase T_1, oligomer fractions of the size range of tRNA were thus obtained in 51-86% yields, better than the yields obtained with MS2 RNA itself where the best yields did not exceed 28% of the total RNA. The larger oligonucleotides remaining could not be recovered from DEAE-cellulose used to fractionate the smaller oligomers. More stringent and different conditions for virus derivatization of fd virus were required. SDS treatment was not used since micrococcal nuclease (MN) was inactive in the presence of SDS. Succinic anhydride in aqueous buffers had no effect on fd, as measured by sucrose gradient sedimentation of DNA of MN-treated fd derivatives. NBS first produced aggregation (increase in S_{20}) and then monomer formation (decrease in S_{20} over native fd) with increasing amounts of reagent to virus. However, MN hydrolysis gave no distinct oligomer patterns on GE, only smears, a result, perhaps, of the decreased solubility of fd at pH 3-4, the required pH for reaction of NBS with tryptophan or tyrosine, even in 7 M urea.

Oxidation of exposed Ap residues in DNA may also occur, even though this reaction is usually carried out at higher temperatures (boiling for 1-2 hours). Work is in progress on the modification of fd in 7 M urea with succinic anhydride, as well as with DTE or DTT.

Significance to Bio-Medical Research and the Program of the Institute:

Use of substituted polyacrylamide for GE of large oligonucleotides gave no increase in capacity over the usual gel under the conditions tested. While GE is probably the best procedure for the fractionation of large oligonucleotides, substitution with charged groups does not improve its low capacity, an obstacle to preparative GE capable of providing mg amounts of purified large oligonucleotides.

As an alternative approach, modification of whole virus followed by partial nuclease hydrolysis may produce a reduced number of large oligonucleotides compared to the number of such compounds obtained from the free nucleic acid. A reduction in the number of large oligonucleotides would facilitate their fractionation and characterization for various purposes, ranging from sequence determinations to tests for messenger RNA activity. A systematic search along these lines is in progress.

Honors and Awards: None

Publications:

Rushizky, G. W. and Mozejko, J. H.: Partial hydrolysis of MS2 RNA with RNase U_2 *B. amyloliquefaciens RNase*, or micrococcal nuclease. *Anal. Biochem.* 51: 328-331, 1973.

Serial No. NIAMDD-LNE-11
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Protein-Nucleic acid interaction: Chromatin structure and restriction in eukaryotic cells.

Previous Serial Number: SAME

Principal Investigator: Dr. Robert T. Simpson

Other Investigators: Dr. Ronald Seale and Miss Linda Propst

Cooperating Units: None

Man Years

Total:	1.0
Professional:	0.5
Other:	0.5

Project Description

Objectives: To study the structure and enzymatic bases for the restriction of translation of the major portion of genetic information in nucleated, mammalian cells; and to study the mechanisms which allow transcription of that portion of the genetic message which is appropriate to the function of the particular cell type.

Methods Employed: Using established methodology, nuclei are isolated from mammalian cells, and then chromatin, the diffuse interphase form of the chromosomes, is isolated from these nuclei. The physicochemical properties of the isolated chromatin are studied using standard biochemical techniques, such as selective chemical modification, circular dichroism, absorption spectrophotometry, ultracentrifugation, hydrogen-ion titration, and interaction with reporter compounds.

Major Findings: We have employed acetylation of chromatin with acetic anhydride followed by isolation of histones and determination of the amount of incorporated acetate and its localization in an effort to ascertain the mode of interaction of the five main histones with DNA in native chromatin. Previous reports outlined the methodology for the modification and described isolation of a number of peptides from tryptic digests of the histones of acetylated chromatin. Ambiguities in compositional analysis when peptides were obtained from a mixture of all the histones made determination of the location of modifiable lysyl residues impossible when all histone fractions were digested together.

We have therefore pursued methods for isolation of the modified histones in order to reduce the ambiguity in the determination of unique peptide sequences. Conventional histone fractionation methods such as chromatography on cation exchange celluloses and resins, gel filtration and differential solvent extraction have proven to be unapplicable for the fractionation of the acetylated proteins. Five main histone fractions can, however, be resolved from one another using hydroxylapatite chromatography of the sodium dodecyl sulfate complexes of the modified proteins. The resolved peaks differ in the amount of acetate present per unit mass of protein and appear to be nearly homogeneous by electrophoretic analysis. We are now accumulating sufficient quantities of these purified, modified proteins in order to localize the nonbound lysyl residues of the individual histones in chromatin.

The use of nuclease digestion as a tool for the study of chromatin structure was explored using three different deoxyribonucleases, DNase I, DNase II, and micrococcal nuclease. The amount of digestible DNA differed for each of three enzymes and was also a function of other experimental conditions, including enzyme to substrate ratio, ionic strength and amount of divalent cation present. We concluded that chromatin structure can not be rigidly defined in terms of amount of "accessible" DNA by such experiments, but that other factors, such as the mode of action of the enzymes utilized and the strength of protein-nucleic acid interactions, which are, in part, a function of ionic conditions, must be considered. The technique remains promising as a comparative tool if a given set of experimental conditions are utilized.

Significance to Bio-Medical Research and the Program of the Institute:

Determination of the structure of the eukaryotic nucleoprotein complex, chromatin, is necessary for understanding the mechanism of gene regulation in higher organisms. The current studies should help in determining the mechanism of histone binding to DNA in chromatin. Coupled with our earlier studies of DNA structure in chromatin, they should generate a meaningful model of the structure of the genetic material of animal cells. Of particular importance will be the extension of these structural studies of chromatin structure to repressed and transcribed segments of chromatin separated by ECTHAM-cellulose chromatography (See LNE-15).

Honors and Awards: None

Publications:

Simpson, R. T.: Modification of chromatin by trypsin: The role of proteins in maintenance of DNA conformation. Biochemistry 11: 2003-2008, 1972.

Levy, S. and Simpson, R. T.: Quantitative immunological search for single-stranded DNA regions. Nature, New Biol. 241: 139-141, 1973.

Serial No. NIAMDD-LNE-12
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The large-scale purification of ribonuclease T_1 and T_2 from crude Takadiastase powder.

Previous Serial Number: SAME

Principal Investigator: Dr. Pamela Roddy

Other Investigators: Dr. G. W. Rushizky and Dr. H. A. Sober

Cooperating Units: None

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description

This project is terminated.

Serial No. NIAMDD-LNE-13
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Fractionation of chromatin and the study of nonhistone proteins.

Previous Serial Number: SAME

Principal Investigator: Dr. Gerald Reeck

Other Investigators: Dr. Robert T. Simpson and Dr. Herbert A. Sober

Man Years

Total: 1.3
Professional: 1.3
Other: 0

Project Description:

Objectives: To characterize the histone and nonhistone proteins of condensed and extended chromatin fractions obtained using ECTHAM-cellulose.

Methods Employed: Chromatin is fractionated on ECTHAM-cellulose as described in Reeck et al., (1972). Total protein and histone contents are determined using the Lowry and Reinecke reagents, respectively. Polyacrylamide gel electrophoresis, both in acidic-urea and SDS-phosphate buffers, is used for detailed examination of proteins. Chromatin proteins are fractionated using standard preparative procedures.

Major Findings: Early and late eluted chromatin fractions provided by ECTHAM-cellulose chromatography have been shown to be more condensed and more extended, respectively, than unfractionated chromatin. Depsite these striking differences in physical properties between early and late eluted fractions, the differences in protein contents are rather subtle. Condensed and extended chromatin fragments contain comparable amounts of all histones, except for histone F1. There is approximately 25% as much of this histone in the extended fraction of rabbit liver and calf thymus chromatins as in the condensed fractions of each chromatin. This, in large part, accounts for the lower histone/DNA mass ration of 0.8 in the extended chromatin fractions as compared to the value of 1.1 to 1.2 in the condensed fractions.

Extended and condensed calf thymus chromatin fractions contain comparable amounts of nonhistone proteins (0.2 g/g DNA), but extended rabbit liver chromatin has considerably more nonhistone than the condensed fraction (2.5 g/g DNA compared to 1.0 g/g DNA). In addition to these quantitative differences, there are clear-cut qualitative differences between the nonhistones of condensed and extended fractions of rabbit liver chromatin. The most striking of these is the occurrence of a particular, rather high molecular weight nonhistone protein, found exclusively within the extended fraction. Differences are also observed between the nonhistones of extended and condensed fractions of rabbit kidney and HeLa chromatins. These differences appear to be specific for each chromatin.

Because of the correlation some investigators have drawn between histone acetylation and phosphorylation and gene activation, and because of the possible enrichment of transcribed DNA sequences in extended chromatin, we have examined the histones of condensed and extended chromatin fractions of rabbit liver and calf thymus for the amounts of acetylated and phsophorylated histone molecules. In each chromatin, the extended and condensed fractions have identical amounts of the modified histones in comparison to the parent histone molecules.

The especially prominent appearance of a particular nonhistone protein in the extended fraction of rabbit liver chromatin has prompted us to pursue the isolation of this protein. A remarkable purification can be obtained by extracting the chromatin with 1.5 M NaCl. This removes most of the histones from DNA, but, of the histones, the protein of interest is almost exclusively extracted. Dialysis of these extracted proteins against 0.01 M Tris•Cl, pH 8.0, gives a considerable further purification since the histones precipitate and the nonhistone is left in solution. The nonhistone can be further purified to a state of apparent homogeneity by chromatography on DEAE-cellulose. Thus, by this relatively simple procedure and without the use of harsh conditions, we have isolated a nonhistone protein with the interesting property of being exclusively associated with the extended fraction of rabbit liver chromatin.

Significance to Bio-Medical Research and the Program of the Institute:

No problem is more central to gaining an understanding of regulatory phenomena in both normal and disease states than the control of gene expression in eukaryotic cells. A direct approach to this problem is the fractionation of chromatin into functionally distinct nucleoprotein fractions and the study of the physical and chemical properties of the fractions. We have taken this route in developing a fractionation scheme with ECTHAM-cellulose. The isolation of a nonhistone, exclusively associated with the putatively transcribable fractions of rabbit liver chromatin, may be a particularly fruitful course of investigation since many workers now believe that the nonhistone chromatin proteins may possess the specificity which some chromatin constituent must possess in order to allow the selective transcription of a limited portion of the genome.

Honors and Awards: None

Publications:

Reeck, G. R., Simpson, R. T., and Sober, H. A.: Resolution of a spectrum of nucleoprotein species in sonicated chromatin. Proc. Nat. Acad. Sci. U.S. 69: 2317-2321, 1972.

Simpson, R. T. and Reeck, G. R.: A comparison of the proteins of condensed and extended chromatin fractions of rabbit liver and calf thymus. Biochemistry (in press).

Levy, R., Levy, S., Rosenberg, S. A. and Simpson, R. T.: Selective stimulation of nonhistone chromatin protein synthesis in lymphoid cells by phytohemagglutinin. Biochemistry 12: 224-228, 1973

Serial No. NIAMDD-LNE-14
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1973

Project Title: Properties of folic acid γ-glutamyl carboxypeptidase.

Previous Serial Number: SAME

Principal Investigator: Dr. Gerald Reeck

Other Investigators: Mr. Howard Bakerman

Cooperating Units: None

Man Years

Total:	1.3
Professional:	0.3
Other:	1.0

Project Description

Objectives: To develop a rapid enzymatic assay for folate conjugase and to isolate a substantial quantity of an appropriate substrate.

Methods Employed: Conjugase activity is assayed by microbiological techniques and DEAE-cellulose thin layer chromatography of reaction mixtures. Folates are assayed by microbiological techniques. Enzymatic properties of conjugase are studied using the DEAE-cellulose thin-layer chromatographic system. The isolation of folates is accomplished using column chromatographic methods.

Major Findings: The availability of a rapid enzymatic assay is of crucial importance in isolating an enzyme. Not only can a considerable amount of time be lost in waiting for the results of a time consuming assay, but in the case of unstable enzymes, considerable enzymatic activity can be lost as well. For this reason, we have concentrated during the last year on the development of a rapid assay for folate conjugase, an enzyme we would like to isolate. The standard assay, which measures the amount of growth of a bacterial culture which can be supported by the sample to be assayed, is very time consuming and requires an overnight incubation period before the results can be evaluated. In the assay developed, a conjugase reaction mixture is subjected to DEAE-cellulose thin layer chromatography whereby the folate used as substrate is cleanly separated from the product of digestion, which has fewer glutamyl residues. Semi-quantitative results obtained by visual estimation of fluorescent intensities of the folate spots following spraying with appropriate reagents are sufficiently accurate for assay of column fractions. Excellent quantitation can be obtained by fluorescence scanning of the chromatograms.

The technique of DEAE-cellulose thin layer chromatography is also valuable in studying the mechanism of action of conjugases. Using the major folates from yeast and *P. cerevisiae,* we have shown that the conjugases from chick liver and pancreas differ both in the final product of their hydrolysis and in the production of intermediate forms during the reaction. The chromatographic system is also useful in assessing the purity of folates and in the qualitative identification of folates.

Both for use as a substrate in folate conjugase assays and because of its intrinsic interest, the large-scale purification of the major folate from *Pediococcus cerevisiae* has been undertaken. Studies on this folate, which was isolated by Mr. Bakerman some years ago, indicate that it probably has four glutamyl residues, the first folate with this number of glutamic acid units isolated from a natural source. Furthermore, its pteridine ring structure is of the $\underline{N}^5$ formyl tetrahydro form, which imparts considerable stability to the molecule. This stability, which contrasts with the instability of many reduced folates, greatly facilitates the use of *P. cerevisiae* as a source of substrate for folate conjugase.

Significance to Bio-Medical Research and the Program of the Institute:

Differences in biological activity between oligo-glutamyl folates, which are the forms found in nature, and monoglutamyl folates, which have been the subject of intensive study in metabolism, are gradually appearing in the literature. Thus, the isolation and study of the oligo-glutamyl forms and the investigation of their synthesis and degradation *in vivo* are of considerable importance. A good deal of control in folate metabolism may well exist that has so far been unrecognized. Our studies are designed to contribute to the growing awareness of the oligo-glutamyl folates and their role in biological systems.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LNE-15
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Structural analysis of the active and inactive portions of the eukaryotic genome.

Previous Serial Number: SAME

Principal Investigator: Dr. Robert Simpson

Other Investigators: Dr. Ione Polacow, Dr. Gerald Reeck and Miss Linda Propst

Cooperating Units:

Man Years

Total:	2.0
Professional:	1.5
Other:	0.5

Project Description:

Objectives: To determine the physico-chemical basis for the functional differences between the transcribable portion and the remainder of the eukaryotic genome.

Methods Employed: Fractionation of chromatin is performed on a weak anion-exchange cellulose, ECTHAM-cellulose, which had been designed specifically for the chromatography of nucleic acids. Fractions are analyzed by quantitative protein determinations, gel electrophoresis, viscosity, circular dichroism spectroscopy, endogenous enzymatic activity, and melting profile.

Major Findings: We have extended our observations on the structure and function of chromatin fractions obtained by chromatography of sonicated chromatin on ECTHAM-cellulose. A primary effort has been to ascertain the functional capacities of the fractions for both *in vivo* and *in vitro* DNA-dependent RNA synthesis. Determination of the *in vitro* "template activity" of the isolated fractions for either calf thymus nucleoplasmic or *E. coli* RNA polymerase under a variety of experimental conditions has led to low and variable activities with no significant differences between the various fractions. The low activity (relative to protein-free DNA) suggests that the small size of our chromatin pieces, required for chromatographic separation, may preclude the accurate assessment of their functional capacity *in vitro*. In contrast to the lack of differences observed for the separated fractions *in vitro*, we have detected significant differences in their capacity for support of in vivo RNA synthesis. When rats are labeled for ten minutes with tritiated orotic acid, liver chromatin isolated, sonicated and centrifuged to remove dissociated RNA and then chromatographed on ECTHAM-cellulose we find that the late-eluted, low-melting chromatin fractions contain twenty-five times the nascent RNA (relative to DNA content) of the early-eluted, high-melting chromatin segments. Supporting results have been obtained for different times of labeling and with different tissues (e.g., HeLa cells). We feel that this observation, together with that of McConaughy and McCarthy, demonstrates that the late-eluted chromatin is preferentially transcribed, while the early-eluted chromatin segments are relatively repressed in vivo.

The second effort in this project has been to ascertain the solution conformation of chromatin segments which are transcribed (late-eluted) and repressed (early-eluted). The circular dichroism spectra of rabbit liver and calf thymus chromatin fractions differ from those of both DNA and unfractionated chromatin. In both cases, the early-eluted chromatin has an even lower ellipticity at 280 nm than unfractionated chromatin while the last-eluted material has an ellipticity much nearer that of protein-free DNA. The decreased ellipticity in this spectral region of chromatin has been attributed to either the assumption of the C form or to supercoiling. Whichever is the case, it is apparent that repressed chromatin has a structure far more like that of the protein-free nucleic acid. Confirmatory results have been obtained by other physicochemical methods of structure evaluation. Light scattering studies indicate similar particle weights for the two chromatin fractions but demonstrate that repressed chromatin exists in a more compact shape than transcribed chromatin. Similarly, sedimentation of chromatin fractions in either linear 5-50% sucrose gradients or self-generating D_2O gradients leads to differing properties for early- and late-eluted chromatin segments. Repressed chromatin sediments more rapidly than unfractionated (21 S vs 18 S in D_2O) while transcribed chromatin sediments more slowly (9S). Since the chromatin segments are of approximately the same size, such differences in sedimentation must arise from differences in particle shape, the repressed chromatin being more compact in structure while the transcribed sequences are in a more extended conformation.

The current investigations support our contention that ECTHAM-cellulose chromatography separates segments of chromatin which differ in both functional and physico-chemical properties. Early-eluted chromatin is repressed in vivo, and exists in a compact, possibly supercoiled, structure. In contrast, late-eluted chromatin segments are preferentially transcribed in vivo and appear to have a solution conformation more nearly that of isolated DNA. Our experimental results are consistent with models for eukaryotic gene regulation which suggest supercoiling of DNA sequences as part of the mechanism of repression of transcription.

Significance to Bio-Medical Research and the program of the Institute:

Probably the most direct approach to understanding the gross basis for transcriptional regulation in eukaryotic cells is the study of the composition and structure of separated repressed and transcribed segments of the genome. We have developed methodology which seems to allow separation of these two types of nucleoprotein and our current studies have established major differences in both their structure and composition. Extension of these studies to detect more subtle differences in structure between the chromatin species, e.g., mode of histone binding, should allow still more detailed insight into the mechanism of transcriptional regulation and its alterations in development or in disease states.

Honors and Awards: None

Publications:

Polacow, I. and Simpson, R. T.: Circular dichroism spectra of putative transcribed and repressed chromatin. Biochem. Biophys. Res. Commun. 52: 202-207, 1973.

Simpson, R. T.: Structure and function of chromatin. Adv. Enzymol. (in press).

Serial No. NIAMDD-LNE-16
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30,1973

Project Title: Purification of the Hurler corrective factor from normal human urine.

Previous Serial Number: SAME

Principal Investigator: Dr.Pamela Roddy

Other Investigators: Dr. Elizabeth Neufeld (A-LBM)

Man Years

Total:	0
Professional:	0
Other:	0

Project Description

This project is terminated.

Serial No. NIAMDD-LNE-17
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biochemistry of pituitary glycoprotein hormones and the role of pituitary proteolytic enzymes in their secretion and activation.

Previous Serial Number: NONE

Principal Investigator: Dr. Peter G. Condliffe

Other Investigators: Dr. J. A. Gladner, LBP, NIAMDD

Cooperating Units: None

Man Years

Total:	1.0
Professional:	1.0
Other:	0

Project Description

Objectives: During the past decade, the sub-units of the pituitary hormones TSH, LH and FSH have been isolated from human glands and from several species of animal glands. As a result, the peptide sequences are known for the sub-units of b-TSH and b-LH. Partial information from peptide mapping of the human hormones, together with recombination experiments involving their various sub-units strongly suggest that the structures of h-TSH and h-LH are very close to those of the bovine.

The demonstration in several laboratories that the glycoprotein hormones from the pituitary and also from the placenta, possess 2 sub-units, not linked by disulfide, or other covalent bonds, raises several questions about their syntheis and the regulation of their secretion.

1. Are the sub-units, which each have between 100 and 120 amino acid residues, synthesized separately and then somehow combined to yield the active hormone or are they derived from a single chain pro-hormone as is the case with insulin?

2. If the sub-units are synthesized separately, are there, in fact, two pro-hormones each of which is activated and which then are combined to yield the active hormone.

3. Do the pituitary proteolytic enzymes, play a role in the secretion and activation of these hormones?

Methods Employed: Against this background, we plan a detailed investigation of the pituitary proteolytic enzymes and their possible role in the conversion of putative pro-hormonal forms of the glycoprotein hormones to the active forms found in the pituitary and the circulation.

To conduct this investigation purified preparations of the hormones are being isolated from bovine pituitaries. Tried and true methods, devised in this laboratory will be used initially. New affinity techniques developed for isolation of other proteins will then be applied to the preparation of the hormones and the proteolytic enzymes.

Antisera are being prepared against bovine TSH and bovine LH. We plan to fractionate these antisera by affinity techniques using previously isolated preparations of the purified hormones bound to sepharose in order to obtain enriched antibody fractions.

Detection and estimation of the hormones will be performed routinely by radio-immunoassay using the antisera mentioned above and employing the pure hormones as labeled antigens. Preliminary results using the solid-state method of radio-immunoassay, devised by Bates and Garrison, have shown that two old preparations of b-TSH can be employed as standards and as labeled antigens in the immunoassay during the intitial phase of the work.

Standard techniques for the assay of proteolytic activity will initially be carried out utilizing overall substrates such as hemoglobin, casein and other protein preparations, to be followed by the use of synthetic substrates to establish absolute specificity.

Proposed Course of Project: Once the above methods have been established and purified preparations of the hormones isolated, a first step will be to study the action of the pituitary proteases on the sub-units of the hormones. Differences will be looked for in the proteolysis of the isolated sub-units by standard techniques for study of protein degradation (gel electrophoresis, peptide mapping etc). The effect of proteolysis on the immunochemical properties of the hormones and the disaggregated sub-units will also be assessed.

Assuming that affinity chromatographic techniques can be worked out, the isolation of the pure hormones will be undertaken from crude extracts. If pituitary protease activity can be successfully inhibited, a search will be made for pro-hormonal components of the pituitary. By analogy with work on insulins, such components should react with antisera directed against the hormones already isolated. Most partly purified preparations of b-TSH contain multiple components some of which appear to be of higher molecular weight. During gel filtration of crude TSH fractions on G-100, a small amount of TSH activity is usually unretarded. Also, in past experiments in which b-TSH extracts were chromatographed on CM-cellulose about 10% of the biological activity was usually unadsorbed; rechromatography of this fraction, which was of low potency, showed that it differed in many respects from the highly purified TSH fractions that were isolated. These experiments were carried out before the development of radio-immunoassays by Berson and Yalow. At this time (before 1960) these experiments were interpreted as being partly denatured, or aggregated material or possibly as TSH adsorbed to other proteins. With the evolution of current ideas about pro-hormones, these old experiments suggest that pro-hormonal forms of TSH and other glycoprotein hormones are waiting to be found.

For many years research on the regulation of pituitary secretion was hampered by the lack of biochemical tools adequate to the task. This situation has changed in the last decade. Chemical identification of the hypothalamic releasing hormones has provided some of the needed tools; establishment of peptide sequences and primary structure for the known pituitary hormones with the exception of FSH, together with the development of immunochemical techniques for the identification and isolation of the hormones puts us in a position to examine the biochemical events related to synthesis and secretion of pituitary hormones.

At this time we do not know how the hypothalamic hormones act to regulate synthesis and secretion of the pituitary hormones. Our view is that by working back through the post-translational events related to the glyco-protein hormones we may be able to pinpoint where in the synthetic machinery TRH, LRH and FRH really exert their effect.

This is a long-term raison d'etre for our project. More immediately, we feel it sufficient to delineate the biochemical events that occur when the hormones are released from the cell. Eventually, when the methods described above are working, they could be applied to pituitary cell lines in tissue culture now being developed in other labs at NIH and extramurally.

So far as NIAMDD research is concerned, the Institute is responsible for support in endocrine diseases. The glycoprotein hormones of the pituitary include the gonadotropins which are key hormones in the regulation of fertility. Better understanding of the biochemistry of glycoprotein hormone secretion will aid in development of methods for c ntr l of human fertility. Many metabolic diseases are affected by malfunctioning of the pituitary. Untangling neurohypothalamic influences from biochemical deficiencies in the pituitary is important for diagnosis and treatment of growth disorders and other metabolic problems. This project is aimed at a central area in endocrine research. Such research on pituitary biochemistry is not now being carried out in NIAMDD.

Honors and Awards: NONE

Publications:

Condliffe, P. C.: Purification and biochemical characterization of thyrotropic hormone. In Berson, S., and Yalow, R. (Eds.): Methods in Investigative and Diagnostic Endocrinology. Amsterdam, North Holland, 1973.

Serial No. NIAMDD-LNE-18
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Metabolic studies on chromatin: Synthesis, assembly and turnover of chromatin constituents.

Previous Serial Number: NONE

Principal Investigator: Dr. Ronald Seale

Other Investigators: None

Cooperating Units: None

Man Years

Total:	1.0
Professional:	1.0
Other:	0

Project Description

Objectives: The theme of this program is the study of functional aspects of chromatin from a biological standpoint. Of immediate interest are problems related to the control mechanism by which synthesis of DNA and histone proteins is coordinated.

Methods Employed: The work this year was centered on two separate studies: 1) Synthesis of DNA in the presence of cycloheximide, and 2) isolation of histone Fl messenger RNA. In both cases, studies were performed on HeLa cells in suspension culture. The first study utilized amounts of cycloheximide sufficient to block protein synthesis below the level of detectability. The DNA synthesized under these conditions was labeled with isotopic thymidine and chromatin was then prepared. In some experiments, prior to administration of cycloheximide and label, the DNA was density-labeled with BUdR. Analysis of chromatin proteins was performed by gel electrophoresis and radioactivity determination of gel slices.

Isolation of histone mRNA involves isolation of total polysomes from synchronized cells in mid S-phase. Polysomal RNA is then extracted with phenol and separated into size classes by sucrose gradient zonal centrifugation. The 5S - 15S region is collected and is subsequently subjected to separation by preparative gel electrophoresis from which the eluates are collected.

Major Findings:

1) DNA Synthesis in the presence of Cycloheximide.

In the presence of cycloheximide (CH) sufficient to block protein synthesis below detectability, DNA synthesis in HeLa cells proceeds at a linear rate of 20% of control for about one hour. That the decreased rate of DNA synthesis is more likely due to a lesion in protein synthesis than interference with replication by cycloheximide was demonstrated by identical results from experiments utilizing puromycin.

The DNA synthesized during CH blockade of protein synthesis was demonstrated by several criteria to be a continuation of the normal replication process and not a repair phenomenon. CH•DNA synthesis is abolished by hydroxy-urea which has been reported not to affect repair replication. Radioactivity incorporated either before or after administation of CH sediments with the bulk of DNA which cosediments preferentially with heavy DNA, indicating covalent linkage to recently replicated DNA.

It was surmised that DNA synthesized in the absence of protein synthesis should be free of chromatin proteins and thus should be more susceptible to nuclease attack than normal chromatin DNA. By use of pancreatic DNase I digestion, CH•DNA was demonstrably more susceptible to enzymatic hydrolysis than controls, but less so than free DNA.

This intermediate value of "digestibility" led to the suspicion that non-bound chromatin protein pools may be present. Cells were pulse-labeled with H^3-leucine 5 minutes and chased by addition of CH. In the ensuing 60 minutes, both histone and nonhistone proteins in chromatin increased in specific activity by 30%. Polyacrylamide gels sliced for radioactivity assay revealed no preferential incorporation of specific histones or nonhistones.

2) Isolation of histone messenger RNA.

Current efforts are directed toward isolation and characterization of histone messenger RNA, notably histone F1 mRNA. Histone F1 is chemically distinct from other histones by virtue of its high lysine content: it has a molecular weight approximately twice that of other histones and it is markedly more antigenic than the other histones. Coupled with the size differentiation of histone messengers from the general RNA population, their appearance at a discrete interval in the cell cycle, sensitivity to inhibitors of DNA synthesis, and reported lack of 3'terminal polyadenylates, histone messengers provide a vehicle for study of control of histone synthesis, especially with regard to DNA synthesis. Initial efforts will be directed toward assessment of purity of messengers which have been isolated electrophoretically and proof as messengers by *in vitro* synthesis and peptide mapping. Next, efforts will be directed toward construction of a DNA reverse transcript, and by hybridization of the reverse transcript to HnRNA, and isolation of the nuclear RNA species which give rise to histone messenger RNA.

Significance to Bio-Medical Research and the Program of the Institute:

Understanding of control mechanisms in eukaryotic cells is of fundamental importance to progress in basic life sciences and ultimately to applications in medicine. To date, no system of control in eukaryotes is understood nearly as well as pro-karyotic mechanisms of induction and repression of expression of macromolecules. Histone synthesis is a temporal, induction-repression phenomenon which occurs during the cell cycle of all eukaryotes. This process is linear and unidirectional and is a prerequisite for cell division. Information relevant to this fundamentally important control mechanism would be of value not only in understanding other eukaryotic controls but in analogous processes of gene expression during development and aberrant control mechanisms which may cause disease.

Honors and Awards: None

Publications: None

ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Carbohydrate Metabolism

A. Mucopolysaccharidoses. The use of fibroblasts cultured from the skin of patients with various genetic disorders has provided a means for the identification of the previously described "corrective factor" for the Hunter syndrome as a sulfoiduronate hydrolase. This enzyme is responsible for the degradation of the abnormal amounts of dermatan and heparan sulfate which accumulates in the tissues of these patients. An analogous enzyme, β-glucuronidase, has been shown to be markedly diminished in a newly described mucopolysaccharidosis; in this case, bovine β-glucuronidase serves as a corrective factor.

I-cell disease is a disorder of connective tissue in which the hydrolytic enzymes are found extracellularly instead of within lysosomes. An explanation for this phenomenon has been found in that the secreted hydrolases lack the appropriate "recognition marker" which permits the cells to identify them prior to uptake from the medium and packaging within the lysosomes. This "recognition marker" is thought to be a carbohydrate moiety since periodate oxidation transforms a normal hydrolase which is captured efficiently by the cells, into one which resembles an I-cell enzyme (i.e., one which is not taken up). A similar biochemical defect underlies a clinically milder disease, pseudo-Hurler polydystrophy. [E. F. Neufeld]

B. Chitin and Glycogen Synthetase. Previous reports have described the development of an hypothesis to explain the mechanism of initiation of cell division in yeast. Briefly, it was proposed that an activating factor transforms inactive chitin synthetase (zymogen) into active enzyme only at certain sites on the cell surface, thus explaining the localization of the chitin septum. A necessary assumption was that activating factor and zymogen reside in different organelles. An effort was therefore made to isolate the organelles which contain the activating factor. This study culminated in the preparation of a highly purified vacuole fraction, greatly enriched in chitin synthetase activating factor (20 to 25-fold on a protein basis) and essentially free from zymogen. The latter was found in another, membrane-rich fraction, which was devoid of activating factor.

The vacuole fraction was further characterized by phase-contrast and electron microscopy and by the determination of different enzymatic activities. A neutral protease found in an extract from crude particles was enriched about as much as the activating factor. The paralleled purification of neutral protease and chitin synthetase activating factor supported the identity of these two activities. Further study showed that the inhibition of both activities (protease and activating factor) were

During the past decade this laboratory among others has made frequent use of glycerol as a means of stabilizing enzymes which could not have been purified without this reagent. The stabilizing effect makes glycerol an extraordinarily effective adjunct to enzyme purification when used at concentrations in the range of 20 to 50 percent by volume. The effect of glycerol on yeast aldehyde dehydrogenase is to change the confirmation of the enzyme in a manner such that it binds both substrates, aldehyde and pyridine nucleotide, far better than when in aqueous solution. [S. L. Bradbury] There is new evidence that the phenomenon displayed by aldehyde dehydrogenase is a general one, i.e., the presence of a polyhydric alcohol results in a change in the kinetic parameters of each of the 16 other enzymes examined. [J. L. Myers] Data obtained by the use of kinetics, fluorescence probes, titration of thiol groups and difference spectra are consistent with the conclusion that high concentrations of the polyhydric alcohols result in a reversible change in protein conformation.

A group of enzymes, glutathione transferases, present in mammalian livers, catalyze the first step in mercapturic acid formation, the conjugation of glutathione and any of a large number of compounds bearing anucleophilic group such as halide, nitrite, epoxide and sulfate. Three, possibly four, enzymes have now been isolated in a homogeneous state which catalyzes each of these reactions. [T. A. Fjellstedt, W. G. Habig, M. J. Pabst] P-Nitrobenzyl chloride served as substrate for each enzyme. Comparison of the four isolated enzymes with a variety of substrates revealed each to be active with more than one class of substrate, although the extent of enzymatic activity varied widely.

As part of a major means of detoxication, these enzymes are being studied with regard to their relationship to each other as well as the range of substrates. [W. B. Jakoby]

I. Studies on the Synthesis and Degradation of Nucleic Acids. In the early months of the year being reported, work in collaboration with Professor Winocour was completed. The nature of the defective simian virus 40 DNA arising upon serial undiluted passage of the virus in monkey kidney cells was studied in some detail by means of a bacterial restriction DNase from *Hemophilus influenzae*. These studies confirmed the earlier finding that the defective DNA contains regions in which viral DNA has been lost and replaced by host sequences. With the particular defective DNA studied, the pattern of fragments, produced by cleavage with the restriction enzyme, is completely different from the pattern obtained with the original, nondefective virus. Thus the viral DNA has been drastically altered over most of the molecule DNA-DNA hybridization of the fragments generated by the restriction endonuclease with both SV 40 and monkey DNA permits a more accurate estimate of the extent of substitution. At least 25 percent of the original sequences have been substituted by monkey DNA. Quantitative evaluation of the hybridization data revealed further that the host sequences incorporated into the viral genome are of two types: namely, the repetitive and unique DNA sequences.

quantitatively identical. Thus, there is now a very sound basis for the belief that protease and activating factor are one and the same activity.

The purification and characterization of the heat-stable protein which inhibits the chitin synthetase activating factor, has been carried further. By the use of preparative polyacrylamide gel electrophoresis, a preparation has been obtained which is homogeneous by the criterion of analytical disc gel electrophoresis. The molecular weight of the protein is between 11,000 and 13,000, as determined by polyacrylamide gel electrophoresis in the absence or in the presence of sodium dodecyl sulfate. The protein was found to yield a single amino-terminal amino acid, which was identified as threonine.

Studies on the purification of the different forms of yeast glycogen synthetases have been continued. The procedure previously found for the separation of the glucose-6-phosphate independent (I) and glucose-6-phosphate dependent (D) form has yielded preparations of each form free from the other and from glycogen. The D form of glycogen synthetase has now been purified to homogeneity, as judged by polyacrylamide disc gel electrophoresis. The purified enzyme has a molecular weight of about 300,000 in the ultracentrifuge. Treatment with sodium dodecyl sulfate, followed by polyacrylamide gel electrophoresis, results in the production of subunits of molecular weight approximately 80,000. Although two different subunits, with very close mobilities, were found, there are indications that a single subunit may have been originally present. The other subunit would have arisen from it, perhaps by proteolysis. [E. Cabib]

C. Particulate Enzymes of Carbohydrate Metabolism. 1,3-Diphosphoglycerate, one of the major intermediates in the biological conversion of carbohydrates into adenosine triphosphate, has been shown to be capable of phosphorylating glucose and other sugars and alcohols without the intermediary participation of nucleotides. By means of isotopically labeled compounds it has been found that only the acid anhydride phosphate group is transferred. Acceptor specificity and kinetic studies of the liver and kidney microsomal membrane enzyme which catalyzes the reaction have been carried out. [M. Stetten]

D. Inositol Biosynthesis. Evidence has been obtained which indicates the formation of 2-inosine-1-P as an intermediate in the cyclase reaction whereby glucose-6-P is converted to inositol. Incubation of the partially purified testis enzyme with glucose-6-P and NAD, followed by reduction with tritiated borohydride, resulted in the formation of radioactive myoinositol and scyllitol which were isolated by carrier dilution. Since these cyclitols are 2-epimers, their precursor keto inositol phosphate must have been 2-inosinose-1-P. An unexpected result of this study was the finding that both cyclitols isolated from the boiled enzyme system were also labeled, but to a lesser extent, suggesting that 2-inosinose-1-P is a tightly bound intermediate that prolonged dialysis fails to remove from the enzyme. [F. Eisenberg, Jr.]

E. Glycoprotein Metabolism. Asialoglycoprotein receptor sites on the plasma membranes of mammalian liver cells have been solubilized with nonionic detergents and have been purified extensively by affinity chromatography. Binding studies on the purified material indicate the presence of at least two sites with markedly different affinities. As was true with intact membranes, the binding reaction appears to be essentially irreversible, to require calcium and to be dependent upon the presence of sialic acid residues on the solubilized receptor protein.

Studies with freshly prepared hepatocytes isolated from perfused livers have yielded preparations which bind and transport the bound asialoglycoprotein into the cell. Transport, which is time- and temperature dependent, also appears to be energy dependent in that it is markedly diminished in the presence of metabolic inhibitors such as cyanide and azide.

Continuing studies on the structural determinants of the glycose moiety involved in the binding process have revealed that all of the requirements for binding are located in the carbohydrate chain. Intact oligosaccharides isolated from the desialylated glycoproteins by prolonged proteolysis and released from the amino acid linkage point by specific enzymatic hydrolysis, retain the major portion of their original binding capacity. [G. Ashwell]

F. Immunoglobulin Biosynthesis. Freshly isolated rabbit popliteal lymph node cells synthesize and secrete principally immunoglobulin G together with a relatively small amount of immunoglobulin M. After incubation of such cells in tissue culture for 48 hours, [^{3}H]immunoglobulin G production decreased markedly and approached zero. On the other hand, [^{3}H]immunoglobulin M production increased 5- to 10-fold. Thus, at 48 hours and thereafter, > 90% of the [^{3}H]immunoglobulin was accounted for as [^{3}H]immunoglobulin M. The enhanced [^{3}H]immunoglobulin M production is dependent on the presence of fetal calf serum during tissue culture incubation. Fluorodexoyuridine, an inhibitor of DNA synthesis, abolished the emergence of enhanced [^{3}H]immunoglobulin M production when added to cells during latent period but the inhibitor was without effect on immunoglobulin secretion when incubated with cells after enhanced [^{3}H]immunoglobulin M production had already emerged. The results indicate that the cellular source for the enhanced immunoglobulin M secretion cannot be the original immunoglobulin G secreting cells but may be derived from some of the original immunoglobulin M secreting cells or, alternatively, may be derived from cells that are quiescent in the freshly isolated population.

Rabbit lymph node cells incubated in a serum-free medium exhibit a relatively low level of incorporation of [^{3}H]thymidine into DNA. Fetal calf serum stimulated this incorporation as much as 65-fold.

The emergence of enhanced immunoglobulin M production was markedly inhibited when either concanavalin A, phytohemagglutinin or pokeweek mitogen were incubated with such cells in tissue culture. In contrast, bacterial endotoxin increased immunoglobulin M production about 2-4-fold. [M. Kern]

G. Hormone-Dependent Differentiation of Mammary Gland. Prolactin administration to mature virgin mice leads to proliferation of epithelium of the mammary gland. In organ culture, prolactin is not mitogenic, but insulin and insulin-free serum are. These observations are reconciled by the finding that insensitive epithelium is sensitized to insulin and/or serum by prolactin treatment *in vivo*. Thus, it appears that prolactin acts indirectly as a mitogen by rendering the cells susceptible to the aforementioned mitogens.

Epithelial cells from freshly isolated mammary glands of mature virgin mice make minute amounts of casein and exhibit low levels of lactose synthetase A-protein (a UDP-galactosyltransferase) activity. They have barely detectable lactose synthetase B-protein (α-lactalbumin) activity. When cultured in the presence of insulin, hydrocortisone and prolactin, mammary explants from mature virgin mice develop the capacity to synthesize these milk proteins asynchronously. Casein synthesis and A-protein activity reach their maxima after 72 hours of exposure to the hormones and 24 hours before the emergence of B-protein activity. Estradiol-17β, or prolactin, priming of mature virgin mice reduces the time required for the development of B-protein activity in the mammary explants, while the time course of casein synthesis and appearance of A-protein activity is not appreciably affected. As a result, the secretory proteins, casein and α-lactalbumin, develop synchronously. This represents a heretofore undiscovered manifestation of the influence of prolactin in this system.

The epithelial cells from mammary glands of mature virgin mice synthesize increasing levels of casein and α-lactalbumin when cultured in the presence of insulin, hydrocortisone and prolactin. When DNA synthesis is blocked in these cultures by the addition of cytosine arabinoside or 5-fluorodeoxyuridine, augmented milk-protein production does not occur. The effects of cytosine arabinoside are prevented by simultaneous addition of deoxycytidine. Neither cytosine arabinoside nor 5-fluorodeoxyuridine have a postmitotic inhibitory effect on casein or α-lactalbumin synthesis. The insulin-induced increase in the combined activities of the intracellular proteins glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase is not affected by either inhibitor. Such a necessary coupling of secretory protein production to DNA synthesis or mitosis or both no longer exists in explants from midpregnant mice. Most of the epithelial cells of this tissue have already undergone their "critical mitosis" *in vivo*. [Y. J. Topper]

H. Enzymatic Utilization of Model Compounds. Investigations concerning the reactivity of a variety of chemical groupings in enzyme catalyzed reactions have been carried out. These studies have continued a 2-fold emphasis on the metabolic utilization of a variety of model compounds bearing specific functional groups and, as well, on those features of proteins responsible for specific catalysis.

Data was also obtained suggesting that the events leading to formation of defective, substituted SV 40 DNA may be random. [M. F. Singer]

J. Biosynthesis of Sulfur-Containing Nucleotides. The sulfur transferase system which forms 4-thiouridine in tRNA is comprised of two enzymes. The first enzyme in the reaction, Factor A, has been purified to homogeneity as determined by disc gel electrophoresis with tRNA in the running buffer to take advantage of the enzyme-tRNA complexing. Such complexing does not require added ATP or Mg as demonstrated with sucrose gradients or filter binding in addition to electrophoresis.

Factor C, the second enzyme in the sequence, is unstable and undergoes an irreversible loss of bound pyridoxal phosphate. The best preparations to date still contain about eight component proteins. However, this enzymatic activity appears to be specific for 4-thiouridine thiolation; careful studies have failed to show any formation of the 2-thiocytidine analogue. Despite reports that spermidine binds specifically to the 4-thiouridine region of tRNA, no evidence could be obtained to indicate a role for this base in the partially purified sulfur transferase system [M. N. Lipsett]

K. Studies on the lac Transport System in E. coli. In an attempt to provide an affinity column for the isolation and purification of the lac carrier protein, ^{35}S-labeled heptaacetyl-methylgalacturonate-thiogalactoside has been prepared and the nonradioactive form of the compound has been deacetylated and de-esterified to yield the corresponding galacturonide. Galacturonic acid, as a model compound, has been coupled to hexane diamine and to ethane diamine. The corresponding amide-amines have been identified electrophoretically but have not been isolated. [I. Leder]

L. Enzymatic Reduction of Disulfide Linkages in Mammalian Cells. Using rat liver as principal source of mammalian cell fractions, it has been found that the cytoplasm contains a single thiol-disulfide oxidoreductase activity (Enzyme I) which can catalyze interchange reactions between GSH and disulfide groups of both protein (e.g., insulin) and non-protein (e.g., cystine) substrates. The microsomal fraction contains two additional activities (Enzymes II and III) catalyzing similar reactions. However, while II and III can catalyze GSH-insulin interchange, III cannot catalyze interchange reactions between GSH and small non-protein disulfide substrates. The latter enzyme, however, exhibits strong catalysis of the oxidative reconstitution of activity from totally reduced ribonuclease and is, therefore, probably identical with the well-inown disulfide interchange enzyme previously described by Anfinsen and coworkers. Enzymes I and II appear to be without corresponding RNase-regenerating activity. [F. Tietze]

M. Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms. The nature of the intermediates involved in the unfolding of proteins has been studies by a variety of techniques, with globular proteins, a fast phase in the kinetics of unfolding has been shown to be a fairly general phenomenon and to be an implicit prediction of any sequential unfolding model. A "medium pace" process, being between the fast phase (microseconds) and the steady state phase (tens of seconds) of kinetic curves has also been detected under certain conditions. Its significance is not yet clear and is being investigated.

Pepsinogen reacts with tetranitromethane to nitrate 9 of the tyrosine residues in the protein. This derivative is incapable of autocatalytic activation to form a nitrated pepsin. However, it is attacked by added pepsin at the same rate as unmodified pepsinogen to give an enzymically inactive product of unknown structure. [P. McPhie]

Serial No. NIAMDD-LBM-1
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Role of Hepatic Plasma Membranes in the Turnover of Circulating Glycoproteins

Previous Serial Number: NIAMDD-LBM-1

Principal Investigator: Dr. Gilbert Ashwell

Other Investigators: Dr. Jean Hickman, Research Chemist
Dr. Roger Hudgin, Research Chemist
Mr. William Pricer, Research Chemist

Cooperating Units: None

Man Years

Total: 3
Professional: 3
Other:

Project Description:

Objectives:

The major objective of this project has been to ascertain the structural determinants of binding of the circulating glycoproteins by the plasma membranes of the liver.

Methods Employed:

Hepatic plasma membranes are isolated by conventional methods of cell fractionation employing sucrose density gradient centrifugation. Utilizing this tissue, an inhibition assay has been developed to provide a quantitative estimate of the binding efficiency of various glycoproteins and their asialo-derivatives.

Major Findings:

Asialo-glycoprotein receptor sites on the plasma membranes of mammalian liver cells have been solubilized with non-ionic detergents and have been purified extensively by affinity chromatography. Binding studies on the purified material indicate the presence of at least two

Major Findings: (Continued)

sites with markedly different affinities. As was true with intact membranes, the binding reaction appears to be essentially irreversible, to require calcium and to be dependent upon the presence of sialic acid residues on the solubilized receptor protein.

Studies with freshly prepared hepatocytes isolated from perfused livers have yielded preparations which bind and transport the bound asialoglycoprotein into the cell. Transport, which is pure and temperature dependent, also appears to be energy dependent in that it is markedly diminished in the presence of metabolic inhibitors such as cyanide and azide.

Continuing studies on the structural determinants of the glycose moiety involved in the binding process have revealed that all of the requirements for binding are located in the carbohydrate chain. Intact oligosaccharides isolated from the desialylated glycoproteins by prolonged proteolysis and released from the amino acid linkage point by specific enzymatic hydrolysis, retain the major portion of their original binding capacity.

Proposed Course of Project:

1. Further purification of the solubilized receptor sites is continuing with the aim of preparing and characterizing a homogeneous binding protein.

2. Utilizing isolated hepatocytes, it is hoped that the mechanism of macromolecular transport can be delineated.

3. Elucidation of the nature of the secondary determinants (galactose is the primary) of binding by carbohydrate chain to the membranes is being pursued.

Publications:

Morell, A. G., and Ashwell, G.: Tritium-labeling of glycoproteins that contain terminal galactose residues. In Ginsburg, V. (Ed.): Methods in Enzymology. New York, Academic Press, 1972, pp. 205-208.

Van Lenten, L., and Ashwell, G.: Tritium-labeling of glycoproteins that contain sialic acid. In Ginsburg, V. (Ed.): Methods in Enzymology. New York, Academic Press, 1972, pp. 209-218.

Ashwell, G.: Carbohydrate antigens: Coupling of carbohydrates to proteins by a mixed anhydride reaction. In Ginsburg, V. (Ed.): Methods in Enzymology. New York, Academic Press, 1972, pp. 219-222.

Publications: (Continued)

Ashwell, G.: The role of sialic acid in the immunological and biological properties of glycoprotein hormones. 4th International Congress of Endocrinology, 1972. Excerpta Med., in press.

Van Lenten, L., and Ashwell, G.: The binding of desialylated glycoproteins by plasma membranes of rat liver: Development of a quantitative inhibition assay. J. Biol. Chem. 247: 4633-4640, 1972.

Serial No. NIAMDD-LBM-2
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Enzymatic Utilization of Model Compounds

Previous Serial Number: NIAMDD-LBM-2

Principal Investigator: Dr. William B. Jakoby

Other Investigators: Dr. Michael J. Pabst, Staff Fellow
Dr. William H. Habig, Staff Fellow

Cooperating Units: None

Man Years

Total:	4
Professional:	3
Other:	1

Project Description:

Objectives:

These investigations are concerned with the reactivity of a variety of chemical groupings in enzyme-catalyzed reactions. Choices are such as to lead to investigations of the general phenomena of enzyme mechanisms. Particular attention is presently directed to a versatile detoxication system by which glutathione is enzymatically conjugated to a variety of toxic compounds prior to further metabolism and excretion.

Methods Employed:

The approach is at the level of work with isolated purified enzymes and involves their study by chemical and physical methods.

Major Findings:

These studies have continued a two-fold emphasis on the metabolic utilization of a variety of model compounds bearing specific functional groups and, as well, on those features of proteins responsible for specific catalysis.

Major Findings: (Continued)

During the past decade this laboratory among others has made frequent use of glycerol as a means of stabilizing enzymes which could not have been purified without this reagent. The stabilizing effect makes glycerol an extraordinarily effective adjunct to enzyme purification when used at concentrations in the range of 20 to 50 percent by volume. The effect of glycerol on yeast aldehyde dehydrogenase is to change the confirmation of the enzyme in a manner such that it binds both substrates, aldehyde and pyridine nucleotide, far better than when in aqueous solution. [Bradbury] There is now evidence that the phenomenon displayed by aldehyde dehydrogenase is a general one, i.e. the presence of a polyhydric alcohol results in a change in the kinetic parameters of each of the 16 other enzymes examined. [Myers] Data obtained by the use of kinetics, fluorescence probes, titration of thiol groups and difference spectra are consistent with the conclusion that high concentrations of the polyhydric alcohols result in a reversible change in protein conformation.

A group of enzymes, glutathione transferases, present in mammalian livers, catalyze the first step in mercapturic acid formation, the conjugation of glutathione and any of a large number of compounds bearing a nucleophilic group such as halide, nitrite, epoxide and sulfate. Three, possibly four, enzymes have now been isolated in a homogeneous state which catalyze each of these reactions. [Fjellstedt, Habig, Pabst] p-Nitrobenzyl chloride served as substrate for each enzyme. Comparison of the four isolated enzymes with a variety of substrates revealed each to be active with more than one class of substrate althrough the extent of enzymatic activity varied widely.

As part of a major means of detoxication, these enzymes are being studied with regard to their relationship to each other as well as their range of substreates.

Significance to NIAMDD Research:

Studies of enzyme mechanism and of patterns of metabolism relate directly to the fundamentals of all biological activity. Work on glutathione conjugation by enzymes from liver is intimately related to a general mechanism of aromatic oxidation as well as to the biological problem of detoxication of noxious compounds.

Proposed Course of Project:

It is expected that investigations eealing with the above-noted projects will be continued and a study of other model reactions will be initiated.

Publications:

Jakoby, W. B. and Fjellstedt, T. A.: Epoxidases. In Boyer, P. D. (Ed.): *The Enzymes*. New York, Academic Press, 1972, pp 199-212.

Bradbury, S. L. and Jakoby, W. B.: Glycerol as an enzyme-stabilizing agent: Effects on aldehyde dehydrogenase. *Proc. Nat. Acad. Sci.* 69: 2373-2376, 1972.

Fjellstedt, T. A., Allen, R. H., Duncan, B. and Jakoby, W. B.: Enzymatic conjugation of epoxides with glutathione. *J. Biol. Chem.* 248: in press.

Meyers, J. S. and Jakoby, W. B.: Effect of polyhydric alcohols on kinetic parameters of enzymes. *Biochem. Biophys. Res. Commun.*, 51: 631-636, 1973.

Jakoby, W. B., Bradbury, S. L., and Clark, J. F. and Steinman, C. R., Aldehyde dehydrogenase from Baker's yeast. *Methods in Enzymology*, in press.

Serial No. NIAMDD-LBM-3
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the Synthesis and Degradation of Nucleic Acids

Previous Serial Number: NIAMDD-LBM-3

Principal Investigator: Dr. Maxine F. Singer

Other Investigators: Dr. Donald LeBlanc, Staff Fellow
Dr. G. R. KoteswarRao, Visiting Fellow

Cooperating Units: Professor Ernest Winocour
Department of Genetics
Weizmann Institute of Science
Rehovot, Israel

Dr. Lee Rosner
Laboratory of Molecular Biology
National Institute of Arthritis, Metabolism, and Digestive Diseases

Man Years

Total: 2.5
Professional: 2.5
Other:

Project Description:

Objectives:

1. To study DNA metabolism in monkey kidney cells infected with simian virus 40.

2. To investigate the enzymatic mechanism of the restriction-modification system of coli phage P-1.

3. To study the physiological role of polynucleotide phosphorylase in *Escherichia coli*.

Methods Employed:

1. Standard tissue culture procedures for growth of monkey kidney cells and simian virus 40. Investigation of DNA metabolism utilizes preparative and analytical ultracentrifugation, nucleic acid hybridization, radioisotope tracer technology, and polyacrylamide gel electrophoresis. A variety of chemical procedures are used for fractionation of cellular components, DNA, and enzymes.

2. Genetic methods are used to construct appropriate mutants of coli phage P-1 and lysogens thereof. From these mutants DNA is isolated as substrate, and enzymes are tested for and fractionated in standard ways.

3. *E. coli* permeable to nucleotide substrates are prepared by toluene treatment and techniques evolved previously for studying polynucleotide phosphorylase reactions are being used. Mutants deficient in polynucleotide phosphorylase are being used.

Major Findings:

1. In the early months of the year being reported, work in collaboration with Professor Winocour was completed. The nature of the defective simian virus 40 DNA arising upon serial undiluted passage of the virus in monkey kidney cells was studied in some detail by means of a bacterial restriction DNase from *Hemophilus influenzae*. These studies confirmed the earlier finding that the defective DNA contains regions in which viral DNA has been lost and replaced by host sequences. With the particular defective DNA studied, the pattern of fragments, produced by cleavage with the restriction enzyme, is completely different from the pattern obtained with the original, nondefective virus. Thus, the viral DNA has been drastically altered over most of the molecule. DNA-DNA hybridization of the fragments generated by the restriction endonuclease with both SV40 and monkey DNA permits a more accurate estimate of the extent of substitution. At least 25% of the original sequences have been substituted by monkey DNA. Quantitative evaluation of the hybridization data revealed further that the host sequences incorporated into the viral genome are of two types: namely, the repetitive and unique DNA sequences.

Data was also obtained suggesting that the events leading to formation of defective, substituted SV40 DNA may be random.

2. The work on P-1 restriction and modification enzymes is being carried out in collaboration with Dr. Lee Rosner of this Institute. The work is, at this writing, in an early stage and no findings have been made.

3. The work on the physiological role of polynucleotide phosphorylase was begun in January and thus far no significant findings have been made.

Significance to NIAMDD Research:

Using the methodology and approaches developed and refined in bacterial studies, rigorous studies of nucleic acid metabolism is animal systems are now possible. Studies with viruses are expected to lead to an understanding of the viruses themselves, but also of the nucleic acid reactions in the normal cell. The studies on substituted, defective SV40 have 2 broad implications:

First they raise the possibility of transduction experiments in animal systems and Second, the formation of the substituted molecules provides an interesting approach to the reactions accompanying viral infections.

Proposed Course of Project:

Work with simian virus 40 will proceed in two ways. We will investigate DNases capable of cleaving SV40 DNA (double-stranded circles) in both infected and uninfected cells, and in particular, in cell nuclei. The relation of such DNases to SV40 replication will be studied. In addition, the differences in metabolism of both viral and cellular DNAs during infection at low and high multiplicities will be investigated in order to obtain information concerning the mechanism of formation of substituted SV40 DNA.

The work on P-1 modification and restriction will be directed toward confirming the genetic analysis by enzymatic analysis. Purification of normal and mutant proteins will follow.

The mechanism of action of polynucleotide phosphorylase is reasonably well understood. However, essentially nothing is known about the physiological role of this enzyme. Our studies will try to determine the nature of polymers made by this enzyme in toluenized cells, under a variety of conditions known to alter RNA metabolism.

Publications:

Holmes, R. K., and Singer, M. F.: Purification and Characterization of Adenylate Kinase as an Apparent ATP-Dependent Inhibitor of RNase II in *E. coli*. *J. Biol. Chem.* 248: 2014-2021, 1973.

Serial No. NIAMDD-LBM-4
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Naturally Occurring Sulfur Nucleotides: Identification, Chemistry, Biosynthesis and Biological Significance

Previous Serial Number: NIAMDD-LBM-4

Other Investigators: Dr. Beatrice Chen, Visiting Associate

Cooperating Units: None

Man Years

Total:	2.2
Professional:	2.0
Other:	0.2

Project Description:

Objectives:

1)To explore the two-enzyme sulfurtransferase system which forms 4-thiouridine in tRNA; 2) to study the tertiary structure of tRNA as it is reflected in the accessibility of its No. 8 uridine to thiolation; and 3) to determine, if possible, the role of 4-thiouridine in the tRNA molecule.

Methods Employed:

Standard separations methods were used for proteins and nucleic acids, i.e., salt fractionation, phase separation, column chromatography and electrophoresis. Sucrose gradients were used for binding studies, along with Sephadex separations and filter binding methods.

Major Findings:

1. Purification

The first enzyme in the reaction, Factor A, has been purified to two components. Final isolation is feasible using preparative disc gel electrophoresis with tRNA in the running buffer to take advantage of the enzyme-tRNA complexing.

Major Findings: (Continued)

Purification of the second enzyme is more difficult, since it has a tendency to lose its pyridoxal phosphate irreversibly. Our best preparations have about eight components.

2. Factor A forms a complex with tRNA, as demonstrated with sucrose gradients, filter binding and disc gel electrophoresis. The complex does not require added ATP or Mg, but whether these will bind to enzyme-tRNA is not yet known.

3. No indications for other nucleotide thiolations which might use the same second enzyme, Factor C, were found. The 2-thiocytidine system was explored most carefully.

4. In view of reports that spermidine binds specifically to the 4-thiouridine region in tRNA, spermidine-modified tRNA was used in the sulfurtransferase reaction. It proved to be indistinguishable from untreated tRNA as a fulfur acceptor.

Significance to NIAMDD Research:

A specific conformation of tRNA is apparently crucial if the molecule is to be thiolated only in the natural 8th position from the 5' end. Thus this sulfurtransferase system offers a new probe for tertiary structure in tRNA.

Proposed Course of Research:

A large-scale preparation of Factor A, hopefully homogeneous, will be carried out. This should make possible identification of the tRNA intermediate which Factor A produces.

Further purification of the second enzyme will be continued. Interactions with the activated intermediate and/or Factor A will be studied, and the fate of the carbon chain of dethiolated cysteine determined.

The specific thiolation at position 8 *in vivo* is more ambiguous *in vitro*. Whether this is a result of impure enzymes or of variations in the tertiary structure of the tRNA can probably be decided with cleaner enzymes. Site-specific enzymatic thiolations will allow us to proceed with comparisons of modified and unmodified tRNAs to explore thr role of 4-thiouridine in tRNA.

Serial No. NIAMDD-LBM-5
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biosynthesis of Storage and Structural Polysaccharides and Its Regulation

Previous Serial Number: NIAMDD-LBM-5

Other Investigators: Dr. Kuo-Ping Huang, Visiting Fellow
Dr. Rodney Ulane, Staff Fellow

Cooperating Units: None

Man Years

Total: 4
Professional: 3
Other:

Project Description:

Objectives:

This project attempts to make use of polysaccharide biosynthesis, as a tool in the study of certain fundamental cellular processes, i.e., 1) the mechanisms by which morphological changes are initiated in the course of cell development, as exemplified by the formation of a septum in the yeast cell; 2) the regulatory systems for the biosynthesis of storage material, using glycogen in yeast as a model.

Methods Employed:

Several strains of yeast and their mutants are used as experimental organisms.

Each problem is investigated at the molecular, subcellular and cellular levels. Thus, in addition to the conventional methods of enzymology, gradient centrifugation is used to isolate cell organelles and to determine the intracellular distribution of the different component of the system under study. Furthermore, these observations are correlated with others made at the cellular level, using both biochemical methods (variations in enzymes and metabolites) and light or electron microscopy (direct observation or autoradiography).

Major Findings:

1. In early studies a hypotesis was developed to explain the mechanism of initiation of the chitinous primary septum during cell division in yeast. Briefly, it was proposed that an activating factor would transform inactive chitin synthetase (zymogen) into active enzyme only at certain sites on the cell surface, thus explaining the localization of the chitin septum. A necessary assumption was that activating factor and zymogen would reside in different organelles, but the evidence for this was only indirect. An effort was therefore made to isolate the organelles which contain the activating factor. In the supposition that these organelles would be similar or identical to the yeast cell vacuoles, the isolation of the latter was attempted. As communicated in last year's report, this study culminated in the preparation of a highly purified vacuole fraction, which was greatly enriched in chitin synthetase activating factor (20 to 25-fold on a protein basis) and was essentially free from zymogen. The latter was found in another, membrane-rich fraction, which was devoid of activating factor.

The vacuole fraction was further characterized by phase-contrast and electron microscopy and by the determination of different enzymatic activities. The acidic protease found in an extract from crude particles was absent in the purified vacuoles, but a neutral protease was enriched about as much as the activating factor. Some enrichment of a ribonuclease activity was also noted. The paralled purification of neutral protease and chitin synthetase activating factor further supported the identity of these two activities. Previous evidence on this point was based on the finding that the protein inhibitor of the activating factor also inhibited the protease. A more careful study showed that the inhibition of both activities (protease and activating factor) were quantitatively identical. Thus, there is now a very sound basis for the belief that protease and activating factor are one and the same activity. This finding may clarify a certain confusion now existing in the field of yeast proteases. Different groups have isolated enzymes and their protein inhibitors, which bear an impressive resemblance to our protease and inhibitor and may well be identical to them.

Whereas these studies have confirmed the hypothesis that the chitin synthetase zymogen and its activating factor reside in different organelles, the precise localization of the zymogen has not been ascertained yet, because the particulate preparations containing the latter are still very impure. In the supposition that the zymogen may be attached to the plasmalemma, efforts to obtain purified preparation of this membrane are being made, using yeast protoplasts as starting material. The proposed goal is to carry out the lysis of protoplasts in such a gentle fashion, that the structure of most cell organelles will be preserved. This is important because several cell organelles (vacuoles, nuclei, mitochondria) are enclosed in membranes, and if they are disrupted, these membranes will be liberated, and may be difficult to separate from the plasmalemma.

Major Findings: (Continued)

Conditions which lead to the preservation of organelles tend, however, to promote aggregation of the particles between each other, thus hindering their separation. It was found that EDTA tends to cause disaggregation and also disruption, whereas divalent cations have an opposite effect. Therefore the results can be controlled to some extent by a careful balancing of the concentration of EDTA and the polyamine spermine in the lysis medium. It was also found that Ficoll helps in the conservation of vacuoles during lysis. Centrifugation of the lysate in a linear sorbitol gradient used in a rather novel way, provides an efficient one-step separation of several organelles. These studies are being pursued further.

2. The purification and characterization of the hear-stable protein which inhibits the chitin synthetase activating factor, has been carried further. By the use of preparative polyacrylamide gel electrophoresis, a preparation has been obtained which is homogeneous by the criterion of analytical disc gel electrophoresis. The molecular weight of the protein is between 11,000 and 13,000 as determined by polyacrylamide gel electrophoresis in the absence or in the presence of sodium dodecyl sulfate. The protein was found to yield a single amino-terminal amino acid, which was identified as threonine. Preliminary amino acid analyses show a very low content in aromatic amino acids and the probable absence of cysteine. The inhibitor is now being submitted to further themical and physical characterization.

3. Many efforts were done in the past to inhibit chitin synthesis "in vivo", in order confirm the manner in which this polysaccharide participates in the formation of the yeast septum. These efforts mainly involved the use of several antibiotics, called Polyoxins, which are powerful competitive inhibitors of chitin synthetase "in vivo" and have also been found to act "in vivo" on many fungi, but not on yeast. Recent experiments, making use of high concentrations of Polyoxin D in the presence of a synthetic growth medium, showed an abnormal pattern of growth in Saccharomyces cerevisiae. The typical result was the appearance of a mother-daughter cell pair which had lysed, expelling the cell content at the junction between the two cells, the place at which the septum should have developed. This very interesting result is now being pursued further with observation in the electron microscope. Aside from the information it may directly provide about the mechanism of septum formation, it may also suggest which morphological aberrations should be expected in a conditional yeast mutant defective in the chitin synthetase system. The search for such a mutant would thus be facilitated.

4. Studies on the purification of the different forms of yeast glycogen synthetases have been continued. The procedure previously found for the separation of the glucose-6-phosphate independent (I) and glucose-6-phosphate dependent (D) form has permitted to obtain preparation of each form free from the other and from glycogen. Thus it has become possible to compare the sedimentation behavior of the two forms in a sucrose gradient. The

Major Findings: (Continued)

results indicate a small difference in sedimentation coefficients which would be more consistent with a different conformation of the two proteins than with a real difference in their molecular weight.

During the purification of the enzymes, it was observed that the I form is inactivated by cold. The effect can be completely reversed by raising again the temperature. The inactivation is prevented totally by glycogen and partially by UDP-glucose, glucose-6-phosphate, ammonium sulfate and glycerol. Only a small difference in the sedimentation coefficient of the enzyme in sucrose gradients at 0 or 30°C was found, suggesting that the change in temperature leads to a conformational modification, rather than to association or dissociation of the enzyme. This is in contrast with the findings reported with many other cold-sensitive enzymes and may have implications for an understanding of the phenomenon of cold-sensitivity.

The D form of glycogen synthetase has now been purified to homogeneity, as judged by polyacrylamide disc gel electrophoresis. The steps used in the purification include DEAE-cellulose column chromatography, ammonium sulfate precipitation, chromatography on Sepharose columns in the absence and in the presence of glycogen. The latter method takes advantage of the specific binding of the enzyme to glycogen in order to separate it from other proteins.

The purified enzyme yields a molecular weight of about 300,000 in the ultracentrifuge. Treatment with sodium dodecyl sulfate, followed by polyacrylamide gel electrophoresis, results in the production of subunits of molecular weight approximately 80,000. Although two different subunits, with very close mobilities, were found, there are indications that a single subunit may have been originally present. The other subunit would have arisen from it, perhaps by proteolysis.

Significance to NIAMDD Research:

The findings related to septum formation in yeast promise to provide for the first time the detailed molecular mechanism for the triggering of a morphological change in the cell. It is hoped that this case will provide a useful model for the general processes of differentiation and cell development.

The regulation of glycogen synthesis, on the other hand, is a model for the problem of energy distribution and storage in the cell. As was remarked in other occasions, there is a striking parallelism between these regulatory mechanisms, as observed in yeast and in mammalian cells. Furthermore, the possibilities of biochemical and genetic experimentation provided by a unicellular organism make of yeast a unique material for these studies.

Proposed Course of Project:

Future efforts will be focussed on several aspects of the chitin synthetase system, namely a) the isolation of plasma membranes and the study of zymogen localization; b) the further characterization of the activating factor inhibitor; c) the purification of the activating factor itself; d) the study of Polyoxin effect on septum formation; e) the isolation of conditional mutants defective in components of the chitin synthetase system.

The purification procedure developed for the D form of yeast glycogen synthetase has provided enzyme in essentially homogeneous form, which will now be studied both from the structural and the kinetic point of view. It is hoped that the purification methods will also be applicable to the I form. The availability of both forms in a purified state will pave the way for the purification and study of the enzymes which interconvert both forms. These enzymes, a kinase and a phosphatase, are involved in the regulation of the glycogen synthetase activity "in vivo" and a study of their properties is essential for an understanding of the regulation of glycogen synthetase in the cell.

Publications:

Huang, K.-P. and Cabib, E.: Separation of the glucose-6-phosphate independent forms of glycogen synthetase from yeast. Biochem. Biophys. Res. Commun. 49: 1610-1616, 1972.

Cabib, E. and Ulane, R.: Chitin synthetase activating factor from yeast, a protease. Biochem. Biophys. Res. Commun. 50: 186-191, 1973.

Cabib, E., Ulane, R., and Bowers, B.: Yeast chitin synthetase. Separation of the zymogen from its activating factor and recovery of the latter in the vacuole fraction. J. Biol. Chem. 248: 1451-1458, 1973.

Huang, K.-P., and Cabib, E.: Yeast glycogen synthetase in the glucose-6--phosphate independent form: a case of cold lability without major changes in molecular size. Biochem. Biophys. Acta 302: 240-248, 1973.

Cabib, E.: Chitin synthetase system from yeast. Methods in Enzymology 28: 572-588, 1972.

Cabib, E., Sonnino, S. and Carminatti, H.: GDP-glucose glycohydrolase from yeast. Methods in Enzymology 28: 979-983, 1972.

Cabib, E., Rothman-Denes, L.B., and Huang, K.-P.: The regulation of glycogen synthesis in yeast. Ann. N.Y. Acad. Sci. 210: 192-206 1973.

Serial No. NIAMDD-LBM-6
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms

Previous Serial Number: NIAMDD-LBM-6

Principal Investigator: Dr. Peter McPhie

Other Investigators: NONE

Cooperating Units: NONE

Man Years

Total:	1
Professional:	1
Other:	

Project Description:

Objectives:

The nature of the intermediates, detected by kinetic studies of protein unfolding, is being investigated by a number of techniques in the hope of revealing something of their structure.

Methods Employed:

Visible and ultraviolet spectroscopy in both manual and recording instruments. Stop flow experiments are performed in an Aminco Morrow apparatus. The Aminco Temperature Jump machine is being converted for operation using ultraviolet optics which are necessary for following many biochemical reactions.

Major Findings:

A fast phase in the kinetics of unfolding of globular proteins has been shown to be a fairly general phenomenon and to be an implicit prediction of any sequential unfolding model. A "medium pace" process, being between the fast phase (microseconds) and the steady state phase (tens of seconds) of kinetic curves has also been detected under certain conditions. Its significance is not yet clear and is being investigated.

Major Findings: (Continued)

Pepsinogen reacts with tetranitromethane to nitrate 9 of the tyrosine residues in the protein. This derivative is incapable of auto catalytic activation to form a nitrated pepsin. However, it is attacked by added pepsin at the same rate as unmodified pepsinogen to give an enzymically inactive product of unknown structure.

Proposed Course of Study:

Kinetic studies of protein unfolding have so far been performed by following the behavior of chromophores intrinsic to the proteins themselves (tyrosine tryptophan and heme residues). These studies are being complemented by the use of extrinsic chromophores which will reflect the behavior of other parts of the molecule. Thus, kinetic studies in the presence of substrates and inhibitors should give information about the folding and unfolding of the active site, while experiments in the presence of pH indicators will reveal changes in proton binding accompanying unfolding, due to the normalization of abnormal groups or by changes in pK's caused by release of less specific interactions.

It is also hoped to perform further studies on the pepsinogen-pepsin system.

Publications:

Summers, M.R. and McPhie, P.: The mechanism of unfolding of globular proteins. Biochem. Biophys. Res. Commun. 47: 831-837, 1972.

McPhie, P.: A spectrophotometric investigation of the pepsinogen-pepsin conversion. J. Biol. Chem. 247: 4277-4281, 1972.

Summers, M. R.: Reaction of N-Bromosuccinimide with reduced ring systems. Apparent protection of GDH with NAD. Biochemistry 11: 3088-3090, 1972.

Serial No. NIAMDD-LBM-7
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Hormone-Dependent Differentiation of Mammary Gland *In Vitro*.

Previous Serial Number: NIAMDD-LBM-7

Principal Investigator: Dr. Yale J. Topper

Other Investigators: Dr. Takami Oka, Dr. Barbara K. Vonderhaar

Cooperating Units: None

Man Years:

Total: 5
Professional: 3
Other: 2

Project Description:

Objectives - To study the molecular and cytological phenomena involved in development of mammary gland.

Methods Employed - Mammary gland explants or mammary epithelial cells are cultured in synthetic media. Synthesis of casein is followed by isolation after the tissue has been pulsed with radioactive precursors. Synthesis of α-lactalbumin is followed by measuring the formation of lactose in tissue extracts. DNA and RNA synthesis are studied using conventional methods. Cell proliferation is determined by cell counts. The accumulation of α-aminoisobutyric acid (AIB) is followed with the use of ^{14}C-AIB or ^{3}H-AIB.

Major Findings - Prolactin administration to mature virgin mice leads to proliferation of epithelium of the mammary gland. In organ culture, prolactin is not mitogenic, but insulin and insulin-free serum are. These observations are reconciled by the finding that insensitive epithelium is sensitized to insulin and/or serum by prolactin treatment *in vivo*. Thus, it appears that prolactin acts indirectly as a mitogen by rendering the cells susceptible to the aforementioned mitogens.

Epithelial cells from freshly isolated mammary glands of mature virgin mice make minute amounts of casein and exhibit low levels of lactose synthetase A-protein (a UDP-galactosyltransferase) activity. They have barely detectable lactose synthetase B-protein (α-lactalbumin) activity. When cultured in the presence of insulin, hydrocortisone and prolactin, mammary explants from mature virgin mice develop the capacity to synthesize these milk proteins asynchronously. Casein synthesis and A-protein activity reach their maxima after 72 hours of exposure to the hormones and 24 hours before the emergence of B-protein activity. Estradiol-17β, or prolactin, priming of mature virgin mice reduces the time required for the development of B-protein activity in the mammary explants, while the time course of casein synthesis and appearance of A-protein activity is not appreciably affected. As a result, the secretory proteins, casein and α-lactalbumin, develop synchronously. This represents a heretofore undiscovered manifestation of the influence of prolactin in this system.

The epithelial cells from mammary glands of mature virgin mice synthesize increasing levels of casein and α-lactalbumin when cultured in the presence of insulin, hydrocortisone and prolactin. When DNA synthesis is blocked in these cultures by the addition of cytosine arabinoside or 5-fluorodeoxyuridine, augmented milk-protein production does not occur. The effects of cytosine arabinoside are prevented by simultaneous addition of deoxycytidine. Neither cytosine arabinoside nor 5-fluorodeoxyuridine have a postmitotic inhibitory effect on casein or α-lactalbumin synthesis. The insulin-induced increase in the combined activities of the intracellular proteins glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase is not affected by either inhibitor. Such a necessary coupling of secretory protein production to DNA synthesis or mitosis or both no longer exists in explants from midpregnant mice. Most of the epithelial cells of this tissue have already undergone their "critical mitosis" in vivo.

Significance to Bio-medical Research and the Program of the Institute - During development of multicellular organisms the progenitors of some types of target cells which respond to different hormones are themselves not sensitive to the hormones. The system described above may be a useful model for studying developmental events which result in the conversion of hormone-insensitive into hormone-sensitive cells. Extension of our studies with insulin-sepharose may further clarify the dynamics of insulin action and those of other polypeptide hormones.

Proposed Course of Project - The molecular mechanism involved in the acquisition of insulin-sensitivity will be studied. Also, the reason why DNA synthesis is required for differentiation of virgin mammary epithelial cells will be investigated.

Honors and Awards: None

Publications:

Oka, T. and Topper, Y. J.: Is prolactin mitogenic for mammary epithelium? Proc. Nat. Acad. Sci. USA 69: 1693-1696, 1972.

Topper, Y. J. and Owens, I. S.: Development of the lactose synthetase system. In Piras, R. and Pontis, H. G. (Eds.): *Biochemistry of the Glycosidic Linkage*. New York, Academic Press, 1972, pp. 483-494.

Oka, T. and Topper, Y. J.: Hormone-dependent accumulation of RER in mouse mammary epithelial cells in vitro. *J. Nat. Cancer Inst.* 48: 1225-1230, 1972.

Oka, T. and Topper, Y. J.: Dynamics of insulin action on mammary epithelium. *Nature New Biol.* 239: 216-217, 1972.

Topper, Y. J. and Oka, T.: Insulin and the mammary epithelial cell membrane. In Mehlman, M. A. and Hanson, R. W. (Eds.): *The Role of Membranes in Metabolic Regulation*. New York, Academic Press, 1972, pp. 341-347.

Topper, Y. J., Oka, T., Owens, I. S. and Vonderhaar, B. K.: Some aspects of mouse mammary gland development from maturity to early pregnancy. *In Vitro* 8: 228-236, 1972.

Vonderhaar, B. K., Owens, I. S. and Topper, Y. J.: An early effect of prolactin on the formation of α-lactalbumin by mouse mammary epithelial cells. *J. Biol. Chem.* 248: 467-471, 1973.

Owens, I. S., Vonderhaar, B. K. and Topper, Y. J.: Concerning the necessary coupling of development to proliferation of mammary epithelial cells. *J. Biol. Chem.* 248: 472-477, 1973.

Serial No. NIAMDD-LBM-8
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Inositol Biosynthesis and Function

Previous Serial Number: SAME

Principal Investigator: Dr. Frank Eisenberg, Jr.

Other Investigators: Dr. Conrad Chen, Dr. Arun Majumder

Cooperating Units: Breast Cancer Section, NCI

Man Years:

Total:	1.5
Professional:	1.5
Other:	0

Project Description:

Objectives - 1) Demonstration of transient intermediates in the cyclization of glucose 6-P to inositol 1-P. If this isomerization is mediated through an intramolecular aldol condensation, driven by an oxidoreduction involving NAD and NADH, two intermediates should be present: 5-ketoglucose 6-P and 2-inosose 1-P. Chemical reduction products, the phosphate esters of glucitol, iditol, myoinositol, and scyllitol, will be sought as evidence for the existence of these intermediates.

2) Studies on the role of myoinositol 1,2 cyclic phosphate (cycInP) in membrane function. Phosphatidyl inositol (PI) in the membrane of intact cells of many types exposed to a variety of external stimuli shows a markedly increased turnover characterized by breakdown to cycInP. Human lymphocytes stimulated by phytohemagglutinin (PHA) to produce increased amounts of DNA show a 20-fold increase in PI turnover within 30 minutes. Based on the hypothesis (Michell and Lapetina, Nature New Biol. 240: 258, 1972) that cycInP derived from membrane PI is the intracellular transmitter of the external stimulus, DNA synthesis will be measured in lymphocytes exposed to CycInP in the presence and absence of PHA.

Methods Employed - 1) Glucose 6-P, NAD, and partially purified testicular cyclase were incubated in Tris buffer at pH 7. Two minutes later NaB^3H_4 was added and phosphate esters were isolated by ion exchange. Free sugars released from the esters by alkaline phosphatase were isolated by paper chromatography and diluted with carrier myoinositol and scyllitol. The cyclitols were isolated and purified by repeated sublimation of the crystalline trimethylsilyl ethers. Specific radioactivity was calculated from the mass, determined by gas-liquid chromatography, and the total radioactivity, determined by liquid scintillation counting. The identical experiment was repeated with replacement of the enzyme with an equal amount of boiled enzyme.

2) CycInP was prepared from commercial inositol 2-P by reaction with dicyclohexylcarbodiimide in pyridine and purified as the cyclohexylammonium salt. The sodium salt was prepared from the latter and incubated with human lymphocytes stimulated to produce DNA and pulsed with tritiated thymidine. DNA was isolated and assayed for ^3H by scintillation counting.

Major Findings - 1) Myoinositol and scyllitol isolated by carrier dilution from the NaB^3H_4 reduced phosphate fraction were found to be radioactive. Since these cyclitols are 2-epimers their precursor keto-inositol phosphate must have been 2-inosose 1-P, a postulated intermediate in the cyclase reaction. This result represents the first time label from a source other than the substrate has been observed in the product of this reaction. An unexpected result of this study was the finding that both cyclitols isolated from the boiled enzyme system were also labeled, but to a lesser extent, suggesting that 2-inosose 1-P is a tightly bound intermediate that enzyme purification, including even dialysis, fails to remove completely.

2) Preliminary results show about 20% augmentation of PHA stimulation of DNA synthesis in lymphocytes by cycInP. As a replacement for PHA, cycInP showed a slight stimulation of DNA synthesis.

Significance to Bio-medical Research and the Program of the Institute - 1) Evidence for a keto intermediate in the glucose 6-P cyclase reaction, taken together with the requirement for NAD, places this enzyme among the internal oxidoreductases, a class of enzymes currently under intense investigation.

2) The presence of phospholipids as structural components of membranes has long been known. That they may play a dynamic role in conveying external stimuli to the interior of the cell is only beginning to be recognized.

Proposed Course of Project - 1) An attempt to demonstrate the other postulated intermediate in the cyclase reaction, 5-ketoglucose 6-P, will be made by the isolation of added carrier iditol from the reduced products. Since iditol is the 5-epimer of glucitol, if it is found to be radioactive it will prove that the parent keto sugar is 5-ketoglucose 6-P. Glucitol, Proposed Course of Project: (Continued)

overwhelmingly labeled in the 1 position since it represents largely unused substrate, can serve no useful purpose in this identification.

2) An assay for tissue levels of cycInP will be developed for the measurement of the compound in lymphocytes (or any other tissue) before and after PHA stimulation. Derivatives of cycInP will be prepared to improve transport into cells, analogous to that requirement for cAMP.

Honors and Awards: NONE

Publications:

Eisenberg, F., Jr.: Inositol 1-phosphate. In Bergmeyer, H. U. (Ed.): Methods of Enzymatic Analysis (3rd German Edition). Weinheim, Verlag Chemie, 1972, pp. 1302-1306.

Eisenberg, F., Jr.: Gas chromatography of phosphorus compounds. In Halmann, M. (Ed.): Analytical Chemistry of Phosphorus Compounds. New York, Wiley Interscience, 1972, pp. 69-91.

Eisenberg, F., Jr.: Gas chromatography of carbohydrates as butaneboronic acid esters. In Ginsburg, V. (Ed.): Methods in Enzymology. New York, Academic Press, 1972, pp. 168-178.

Serial No. NIAMDD-LBM-9
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Enzymatic Reduction of Disulfide Linkages in Mammalian Cells

Previous Serial Number: SAME

Principal Investigator: Dr. Frank Tietze

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Others: 0

Project Description:

Objectives - To investigate enzyme systems of mammalian tissues which catalyze interchange reactions of thiols and disulfides with the view to determining their substrate spectra, subcellular localization, and their relation to cellular function and metabolism.

Methods Employed - The ability of subcellular fractions of mammalian tissue to catalyze thiol-disulfide interchange reactions is determined by a spectrophotometric procedure in which interchange between reduced glutathione (GSH) and a variety of disulfide-containing compounds is coupled enzymatically to the oxidation of reduced pyridine nucleotide through the action of added yeast glutathione reductase. The active enzymic components of these cell fractions are further separated and purified by ion-exchange chromatography.

Major Findings - Using rat liver as principal source of mammalian cell fractions it has been found that

1) The cytoplasm contains a single thiol-disulfide oxidoreductase activity (Enzyme I) which can catalyze interchange reactions between GSH and disulfide groups of both protein (e.g., insulin) and non-protein (e.g., cystine) substrates.

2) The microsomal fraction contains two additional activities (Enzymes II and III) catalyzing similar reactions. However, while II and III can

catalyze GSH-insulin interchange, III cannot, like Enzymes I and II, catalyze interchange reactions between GSH and small non-protein disulfide substrates.

3) Enzyme III from microsomes exhibits strong catalysis of the oxidative reconstitution of activity from totally reduced ribonuclease and is, therefore, probably identical with the well known disulfide interchange enzyme previously described by Anfinsen and co-workers. Enzymes I and II appear to be without corresponding RNase-regenerating activity.

Significance to Bio-medical Research and the Program of the Institute - With the exception of the disulfide-interchange enzyme of Anfinsen and co-workers, little or no information is available concerning the function of the several thiol-disulfide oxidoreductases reported in this and other investigations. It has, however, been variously suggested that these enzymic activities may be associated with such diverse functions as transport and intracellular reduction of cystine, insulin catabolism, regulation of enzyme activity through disulfide interchange with small thiols and disulfides, and in the variation of thiol:disulfide ratios of chromosomal proteins observed during cell division. Conceivably, defects in the catalytic activity of thiol-disulfide oxidoreductases may have important consequences with respect to normal cellular physiology.

Proposed Course of Project - Further investigation of the comparative properties of the soluble and particulate forms of thiol-disulfide oxidoreductases will be carried out. It will be of interest to determine whether the substrate specificity of these enzymes extends to thiols other than glutathione.

Honors and Awards: None

Publications:

Tietze, F., Bradley, K. H. and Schulman, J. D.: Enzymic reduction of cystine by subcellular fractions of cultured and peripheral leukocytes from normal and cystinotic individuals. Pediatric Research 6: 649-658, 1972.

Serial No. NIAMDD-LBM-10
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Biochemical Lesions in the Genetic Mucopolysaccharidoses and Mucolipidoses

Previous Serial Number: SAME

Principal Investigator: Dr. Elizabeth Neufeld

Other Investigators: Dr. Gideon Bach, Dr. Scot Hickman, Mrs. Clara Hall, Mr. James Scott

Cooperating Units: Arthritis and Rheumatism Branch, NIAMDD

Man Years:

Total: 5.5
Professional: 2.5
Others: 3

Project Description:

Objectives - To determine the biochemical defect of genetic disorders of lysosome function, such as mucopolysaccharidoses and mucolipidoses and to use the findings for medical assistance to the patients and their families.

Methods Employed - Fibroblasts are cultured from the skin of normal individuals, of patients with genetic disorders and of relatives of these patients. The fibroblasts are used for measurement of lysosomal enzymes, of mucopolysaccharide accumulation, or of other parameters which are necessary to establish the basic defect in the disorders studied.

Major Findings - 1. The Hunter syndrome was previously shown due to the deficiency of a protein called "Hunter corrective factor", which is necessary for the degradation of dermatan and heparan sulfate. This factor has now been shown to be a sulfoiduronate hydrolase. This project was carried out in collaboration with Dr. Frank Eisenberg.

2. Measurement of α-L-iduronidase in fibroblasts of obligate Hurler heterozygotes has shown that this enzyme is reduced to about half the normal level. However, overlap between level of activity of normal and heterozygote fibroblasts allows this test to be used for carrier detection in selected cases only.

3. In a newly described mucopolysaccharidosis, β-glucuronidase activity is nearly absent; bovine β-glucuronidase serves as corrective factor.

4. I-cell disease is a disorder of connective tissue in which hydrolases are found extracellularly instead of within lysosomes. The defect has been found to be in the hydrolases themselves; these appear to lack a "recognition marker" which would allow the cells to capture them from the medium and package them within lysosomes. A similar biochemical defect underlies a clinically milder disease, pseudoHurler polydystrophy.

5. The "recognition marker" on lysosomal enzymes is thought to be a carbohydrate moiety, since periodate oxidation transforms a normal hydrolase that is captured efficiently by cells, into one which resembles an I-cell enzyme (i.e., is not taken up). There is a wide variation in the ability of N-acetyl-β-hexosaminidase from various sources to be pinocytosed. (Uptake of that enzyme into cells from a Sandhoff disease patient was chosen as a model system for logistic reasons.)

Significance to Bio-medical Research and the Program of the Institute - Correction of the biochemical defect of Hurler and Hunter fibroblasts by replacement of the deficient enzyme has given hope for analogous treatment of patients, not only in these two conditions, but in lysosomal disorders as a class. Clinical trials involving infusions of plasma and leucocytes, or of purified enzymes (carried out in various laboratories throughout the world, though not in our own) have been disappointing for two reasons. After an initial dramatic clinical improvement due to plasma infusion, the patient becomes unresponsive to the plasma; this may be due to the production of antibodies against the relevant enzyme. In the few trials with purified enzymes, the problem has been that the enzyme has gone to the liver (Kupffer cells?) instead of the site where it is needed.

Preparation of pure enzyme may reduce (though probably will not eliminate) the problem of antigenicity. Preparation of enzymes with appropriate recognition markers should ensure their delivery to the desired tissue.

Proposed Course of Project - Simplified methods of purification and assay of iduronidase (Hurler factor) and sulfoiduronate sulfatase (Hunter factor) will be developed. Potential sources of enzyme (e.g., platelets, human liver, placenta) will be examined. The goal is to work out a rapid and efficient preparative procedure which can be readily scaled up so that the enzymes can be turned into drugs for the patients.

The recognition marker necessary for uptake by fibroblasts will be identified. This should also reveal the primary defect in I-cell disease and in pseudoHurler polydystrophy.

Honors and Awards: DHEW Superior Service Award

Publications:

Cantz, M., Chrambach, A. and Neufeld, E. F.: The Hunter corrective factor: Purification and preliminary characterization. J. Biol. Chem. 247: 5456-5462, 1972.

Lie, S. O., McKusick, V. A. and Neufeld, E. F.: Simulation of genetic mucopolysaccharidoses in normal human fibroblasts by alteration of pH of the medium. Proc. Nat. Acad. Sci. USA 69: 2361-2363, 1972.

Neufeld, E. F. and Sweeley, C. C.: Replacement of lysosomal enzymes. Science 178: 648, 1972.

Bach, G., Friedman, R., Weissmann, B. and Neufeld, E. F.: The defect in the Hurler and Scheie syndromes: Deficiency of α-L-iduronidase. Proc. Nat. Acad. Sci. USA 69: 2048-2051, 1972.

Bach, G., Cantz, M., Hall, C. W. and Neufeld, E. F.: Genetic errors of mucopolysaccharide degradation. Biochem. Soc. Trans. 1: 231-234, 1973.

Hall, C. W., Cantz, M. and Neufeld, E. F.: A β-glucuronidase-deficiency mucopolysaccharidosis: Studies in cultured fibroblasts. Arch. Biochem. Biophys. 155: 32-38, 1973.

Hickman, S. and Neufeld, E. F.: A hypothesis for I-cell disease: Defective hydrolases that do not enter lysosomes. Biochem. Biophys. Res. Commun. 49: 992-999, 1972.

Neufeld, E. F., Barton, R. W., Cantz, M., Derge, J. G., Hall, C. W., Kresse, H. and Scott, J. F.: Deficiency of specific proteins in the inborn errors of mucopolysaccharide metabolism. In Aronson, S. M. and Volk, B. W. (Eds.): Sphingolipids, Sphingolipidoses and Allied Disorders. New York, Plenum, 1972, pp. 187-194.

Neufeld, E. F. and Barton, R. W.: Genetic disorders of mucopolysaccharide metabolism. In Gaull, G. E. (Ed.): Biology of Brain Dysfunction. New York, Plenum, 1972, pp. 1-30.

McKusick, V. A., Howell, R. R., Hussels, I. E., Neufeld, E. F. and Stevenson, R. E.: Allelism, non-allelism and genetic compounds among the mucopolysaccharidoses: Hypothesis. Lancet 1: 993-996, 1972.

Neufeld, E. F.: The biochemical basis of the inborn errors of mucopolysaccharide metabolism. In Piras, R. and Pontis, H. G. (Eds.): Biochemistry of the Glycosidic Linkage. New York, Academic Press, 1972, pp. 711-715.

Cantz, M., Kresse, H., Barton, R. W. and Neufeld, E. F.: Corrective factor for inborn errors of mucopolysaccharide metabolism. In Ginsburg, V. (Ed.): Methods in Enzymology, V. 28. New York, Academic Press, 1972, pp. 884-896.

Milunsky, A. and Neufeld, E. F.: The Hunter syndrome in a 46XX girl. New Engl. J. Med. 288: 106, 1973.

Neufeld, E. F. and Cantz, M.: The mucopolysaccharidoses studied in cell culture. In Hers, H. G. and vanHoof, F. (Eds.): Lysosomes and Storage Disease. New York, Academic Press (in press).

Neufeld, E. F., Kresse, H. M., Hall, C. W., Cantz, M. J. and Barton, R. W.: The mucopolysaccharidoses: An illustration of genetic heterogeneity and prospects for therapy. Proc. IV Int'l. Congr. Human Genetics, Paris 1971. (in press).

Neufeld, E. F.: Replacement of genotype-specific proteins in mucopolysaccharidoses. In Desnick, R. J., Bernlohr, R. W. and Krivit, E. W. (Eds.): March of Dimes - Birth Defects. (in press).

McKusick, V. A., Howell, R. R., Hussels, I. E., Neufeld, E. F. and Stevenson, R.: Allelism, non-allelism and genetic compounds among the mucopolysaccharidoses. Corrective factors in nosology, genetics and therapy. Trans. Assoc. Am. Phys. (in press).

Neufeld, E. F.: Uptake of lysosomal enzymes by cultured fibroblasts: Studies of mucopolysaccharidoses and of I-cell disease. In Cox, R. P. (Ed.): Cell Communications. New York, Wiley Interscience (in press).

Tondeur, M. and Neufeld, E. F.: The mucopolysaccharidoses - biochemistry and ultrastructure. In Day, S. B. (Ed.): Molecular Pathology. Springfield, Ill., Charles C. Thomas (in press).

Neufeld, E. F. and Hickman, S.: Replacement of lysosomal enzymes: A complication. In Cusworth, D. S. (Ed.): Prospects for Hereditary Metabolic Disease Control. (in press).

Serial No. NIAMDD-LBM-11
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Membrane Transport

Previous Serial Number: SAME

Principal Investigator: Dr. Irwin G. Leder

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Objectives - To study the *lac* transport system in *E. coli*.

Methods Employed - An overproduction of the *lac* enzymes is induced in two special strains of *E. coli*; a merodiploid carrying a *lac* episome and a strain carrying a defective *lac* transducing phage. Affinity chromatography is used to isolate and purify the *lac* permease protein from cell lysates.

Major Findings - ^{35}S-Labeled heptaacetyl-methylgalacturonate-thiogalactoside has been prepared and the non-radioactive form of the compound has been deacetylated and de-esterified to yield the corresponding galacturonide desired for affinity studies. Galacturonic acid, as a model compound, has been coupled to hexane diamine and to ethane diamine. The corresponding amide-amines have been identified electrophoretically but have not been isolated.

Significance to Bio-medical Research and the Program of the Institute - Elucidation of the role, structure and integration of the *lac* carrier protein in the bacterial membrane will provide insight into the general properties of membranes and in particular how membranes interact with effector molecules in normal and diseased states.

Proposed Course of Project - The galactose-thio-galacturonide ligand will be coupled to ethane diamine and subsequently insolubilized to CNBr activated sepharose. This derivitized sepharose, bearing a high number of ligand groups per ml and having no unamidated amine "arms" will be used to isolate the *lac* carrier protein.

Honors and Awards: None

Publications:

Leder, I. G.: Interrelated effects of cold shock and osmotic pressure on the permeability of the *Escherichia Coli* membrane to permease accumulated substrates. *J. Bacteriol.* 111: 211-219, 1972.

Serial No. NIAMDD-LBM-12
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Differentiation of Lymphoid Cells

Previous Serial Number: SAME

Principal Investigator: Dr. Milton Kern

Other Investigators: Dr. Daniel H. Zimmerman, Dr. Melvin B. Hayes

Cooperating Units: None

Man Years:

Total: 3.5
Professional: 2.5
Other: 1

Project Description:

Objectives - The purpose of this project is to gain insight into the process of differentiation of lymphoid cells with particular emphasis on the cellular conversions and interactions which control the induction of the synthesis and secretion of the major classes of immunoglobulins.

Methods Employed - Lymphoid cells derived from rabbit and mouse were incubated in tissue culture medium and at 24 hour intervals the cells were washed and then incubated again for 90 minutes in leucine-free medium supplemented with ^{3}H-leucine. The resultant secreted ^{3}H-immunoglobulins in the extracellular fluids were analyzed to assess the amount of immunoglobulin M and immunoglobulin G produced. Coprecipitation in the presence of carrier class specific immunoglobulins and class specific antiserums was the fundamental technique used. Molecular sieve chromatography and sodium dodecyl sulfate - acrylamide gel electrophoresis were employed to provide additional verification.

Major Findings - Freshly isolated rabbit popliteal lymph node cells synthesize and secrete principally immunoglobulin G together with a relatively small amount of immunoglobulin M. After incubation of such cells in tissue culture for 48 hours, [^{3}H]immunoglobulin G production decreased markedly and approached zero, in most experiments. On the other hand, [^{3}H]immunoglobulin M production increased 5- to 10-fold. Thus, at 48 hours and thereafter $>$ 90% of the [^{3}H]immunoglobulin was accounted for as [^{3}H]immunoglobulin M. In contrast, lymphoid cells derived from rabbit appendix do not show enhanced [^{3}H]immunoglobulin M production although such cells predominantly secrete [^{3}H]immunoglobulin M when freshly isolated and in decreasing amounts during tissue culture incubation. The enhanced [^{3}H]immunoglobulin M production observed with popliteal lymph node cells is dependent on the presence of fetal calf serum during tissue culture incubation. This requirement cannot be replaced by serum from the same rabbit that was the source of the cells and, indeed, such autologous serum in the presence of fetal calf serum inhibited enhanced [^{3}H]immunoglobulin M production. Fluorodeoxyuridine, an inhibitor of DNA synthesis, abolished the emergence of enhanced [^{3}H]immunoglobulin M production when added to cells during the latent period but the inhibitor was without effect on immunoglobulin secretion when incubated with cells after enhanced [^{3}H]immunoglobulin M production had already emerged. The results indicate that the cellular source for the enhanced immunoglobulin M secretion cannot be the original immunoglobulin G secreting cells but may be derived from some of the original immunoglobulin M secreting cells or alternatively, may be derived from cells that are quiescent in the freshly isolated population.

Rabbit lymph node cells incubated in a serum free medium exhibit a relatively low level of incorporation of [^{3}H]thymidine into DNA. Fetal calf serum stimulated this incorporation as much as 65-fold. On the other hand, rabbit serum from the same animal that was the source of the cells did not effectively support [^{3}H]thymidine incorporation (only a 0-2.7-fold greater incorporation than serum free medium) and such autologous serum was inhibitory when added in conjunction with fetal calf serum. This same behavior for both fetal calf serum and autologous serum was observed when the emergence of enhanced immunoglobulin M production was assessed rather than [^{3}H]thymidine incorporation.

The emergence of enhanced immunoglobulin M production was markedly inhibited when either concanavilin A, phytohemagglutinin or pokeweed mitogen was incubated with such cells in tissue culture. In contrast, bacterial endotoxin increased immunoglobulin M production about 2-4-fold. Concanavilin A did not inhibit the secretion of immunoglobulin M when added to cells which already exhibit enhanced immunoglobulin M production, i.e., at 48 hours in tissue culture. However, if concanavilin A was added at 24 hours or earlier its full inhibitory activity was manifested.

Significance to Bio-medical Research and the Program of the Institute - The abnormal γ-globulins and their subunits that have been observed in serum and urine may be a consequence of aberrations in the secretory mechanism, as well as the synthetic mechanism. Studies from this laboratory have elucidated several new parameters that define synthesis and secretion of normal γ-globulin and provide a means for assessing the similarities and dissimilarities between a normal and abnormal cell.

Arrested differentiation of a cell could lead to the circumstance where such cell multiplies and continues to secrete immunoglobulin of a single specificity in enormous quantities, i.e., as occurs with myeloma. Thus, a knowledge of lymphoid cell differentiation may well be vital in understanding and perhaps coping with a variety of immunological deficiencies.

Proposed Course of Project - Efforts will be directed toward sorting out lymph node cell populations to determine whether more than one cell type is required to observe the emergence of enhanced immunoglobulin M production. The rabbit serum inhibitor will be subjected to purification and characterization. In addition, purification of the fetal calf serum component(s) will be undertaken.

Honors and Awards: None

Publications:

Zimmerman, D. H. and Kern, M.: Synthesis and secretion of γ-globulin by lymph node cells. X. The generation of incompletely synthesized immunoglobulin heavy chains. J. Biol. Chem. 247: 4566-4571, 1972.

Zimmerman, D. H. and Kern, M.: Differentiation of lymphoid cells: The *in vitro* emergence of immunoglobulin M secreting cells. J. Immunol. (in press).

Kern, M.: Antibody synthesis in relation to mammalian membranes. In Jamieson, G. A. and Greenwalt, P. J. (Eds.): Mammalian Cell Membranes. London, Butterworth & Co., 1973 (in press).

Serial No NIAMDD-LBM-13
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Particulate Enzymes of Carbohydrate Metabolism

Previous Serial Number: SAME

Principal Investigator: Dr. Marjorie R. Stetten

Other Investigator: Mr. Paul Goldsmith

Cooperating Units: None

Man Years:

Total: 2
Professional: 1
Other: 1

Project Description:

Objectives - To study the occurrence and properties of membrane bound enzymes of liver and kidney which catalyze the synthesis and hydrolysis of a variety of phosphorylated sugars and sugar alcohols.

Methods Employed - Microsomal membrane enzyme preparations from homogenates of organs of normal and abnormal rats are obtained by differential centrifugation methods and are activated by treatment with such reagents as deoxycholate, Triton X-100, digitonin or hydroxyl ion. Conventional chemical or enzyme assay methods, when applicable, are used for kinetic studies and quantitative measurements. When enzymatic methods are not available, radioactive ^{32}P or ^{14}C incorporation methods are employed. New phosphorylated products are isolated and characterized by chromatographic and chemical methods. Methods for the solubilization and purification of inorganic pyrophosphate-glucose phosphotransferase are being studied.

Major Findings - 1,3-Diphosphoglycerate, one of the major intermediates in the biological conversion of carbohydrates into adenosine triphosphate, has been shown to be capable of phosphorylating glucose and other sugars and alcohols without the intermediary participation of nucleotides. By means of isotopically labeled compounds it has been found that only the acid anhydride phosphate group is transferred. Acceptor specificity and kinetic studies of the liver and kidney microsomal membrane enzyme which catalyzes the reaction have been carried out and a paper reporting the results is in preparation.

Earlier studies of the synthetic activities of microsomal glucose-6-phosphatase with inorganic pyrophosphate as the phosphoryl donor have been extended. The membrane enzyme system has been found to be incapable of catalyzing the introduction of a second phosphate group into sugar or alcohol phosphates.

Significance to Bio-medical Research and the Program of the Institute - The enzymes being studied are capable of using PP_i, 1,3-diphosphoglycerate or carbamyl phosphate instead of ATP for the activation of sugars and sugar alcohols prior to their utilization by animal tissues. The quantitative levels of these membrane-bound enzymes have been found to be particularly susceptible *in vivo* to fasting and to a number of endocrine changes, such as result from diabetes, adrenalectomy, cortisone and insulin administration. Conditions of high glucose levels and increased enzyme activity suggest a physiological function of the PP_i-glucose-phosphotransferase for the synthesis of glucose 6-phosphate in the diabetic animal.

It is hoped that these studies may contribute to a better understanding of normal and abnormal carbohydrate metabolism.

Proposed Course of Project - A developmental study of the properties and activity levels of several membrane bound enzymes in liver and kidney of pre- and postnatal rats is in progress. In this connection attempts will be made to develop an antibody to the enzyme glucose-6-phosphatase. The possible metabolic role of sn-glycerol-1-phosphate will be investigated. We have found an enzymatic method of synthesizing this abnormal isomer of the ubiquitous intermediate, sn-glycerol-3-phosphate.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LBM-14
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mechanism of Action of the Hunter Corrective Factor

Previous Serial Number: None

Principal Investigator: Dr. Frank Eisenberg, Jr.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 0.5
Professional: 0.5
Other: 0

Project Description:

Objectives - Elucidation of the catalytic function of the Hunter corrective factor. Hunter factor will be examined to determine whether the sulfatase activity associated with it is specific for terminal sulfoiduronic residues, the initial point of attack of enzymes which normally degrade mucopolysaccharides.

Methods Employed - Although the liberation of inorganic sulfate by the Hunter factor from both dermatan sulfate and heparan sulfate has been demonstrated, the question of the location of the sulfate group liberated remained unanswered. On the supposition that it was linked to terminal iduronic acid, a method was developed for determination of the iduronic acid liberated by the combined action of L-iduronidase (Hurler factor) and Hunter factor. Owing to the difficulty of determining a terminal group split from a large mucopolysaccharide molecule, a model disaccharide, sulfoiduronosyl anhydromannose sulfate, was used instead. Liberated iduronic acid was reduced to idonic acid, lactonized, and converted into the volatile butaneboronic acid ester and measured by gas-liquid chromatography.

Major Findings - Sulfoiduronosyl anhydromannose sulfate incubated with purified Hurler factor (L-α-iduronidase) or with fibroblast acetone powder extracts from Hunter patients (sulfatase deficient, iduronidase normal) yielded no iduronic acid. Or when the substrate was incubated with purified Hunter factor (sulfatase) or fibroblast acetone powder extracts from Hurler patients (iduronidase deficient, sulfatase normal) again no iduronic acid was released. Combination of the two factors or the two kinds of abnormal acetone powders resulted in liberation of free iduronic acid equivalent to that from normal individuals. The Hunter syndrome is thus a deficiency of the sulfatase specific for desulfation of terminal iduronic acid residues. Until this sulfate is removed, followed by removal of iduronic acid, mucopolysaccharides (heparan sulfate and dermatan sulfate) normally present in cells are not degraded by other lytic enzymes and accumulate to pathological levels.

Significance to Bio-medical Research and the Program of the Institute - Hunter's syndrome, one of the mucopolysaccharidoses, is a metabolic disease. An understanding of the nature of the defect in this disorder can serve as a model for the study of other enzyme deficiency diseases.

Proposed Course of Project - No further work is contemplated.

Honors and Awards: None

Publications:

Bach, G., Eisenberg, F., Jr., Cantz, M. and Neufeld, E. F.: The defect in the Hunter syndrome: Deficiency of sulfoiduronate sulfatase. Proc. Nat. Acad. Sci. USA (in press).

ANNUAL REPORT OF THE LABORATORY OF CHEMISTRY

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

SECTION ON BIOCHEMICAL MECHANISMS

Fluoroimidazoles in Biochemistry

Synthetic methods for the 2-fluoro and 4-fluoro analogs of histamine and histidine have been improved and the sequences shortened considerably. Sufficient stocks of these materials are now available to begin detailed biochemical and pharmacological studies. To date, the following observations have been made: 2-fluorohistamine shows at least 75% of the activity of histamine in stimulating the formation of cyclic AMP in brain slices; 4-fluorohistamine is not an antagonist of histamine in gut-response tests, but is a substrate for histamine-N-methyl transferase; 4-fluorohistidine is nontoxic to mice but has a potentially useful soporific effect; this amino acid is decarboxylated by histidine decarboxylase, is deaminated by amino acid oxidase, and is a potent inhibitor (and weak substrate) for histidine-ammonia lyase; 2-fluorohistidine is neither a substrate nor inhibitor of histidine decarboxylase or of histidine-ammonia lyase. Ribonuclease-S-peptide containing 4-fluorohistidine combines readily with ribonuclease-S-protein, but the adduct shows no enzymatic activity. THRF (thyrotropic hormone-releasing factor) and LHRF (luteinizing hormone-releasing factor) have been synthesized with 4-fluorohistidine in place of histidine; these analogs are inactive. Syntheses of angiotensin and of ACTH with this amino acid analog have been initiated. Numerous other studies are in progress. (K. L. Kirk and L. A. Cohen).

2-Fluoroimidazoles as Affinity Labels

During the past year, the important observation was made that 2-fluoroimidazoles are reactive toward good nucleophiles (with replacement of fluorine), whereas 4-fluoroimidazoles are fully stable. Since syntheses have already been developed for 2-fluorohistamine and 2-fluorohistidine, the possibility now exists for testing these analogs as affinity labels for enzyme and receptor sites which have a specific binding affinity for the imidazole ring. Model experiments have shown that the greatest reactivity toward nucleophiles exists in the neutral pH range, and that these compounds would be acted upon only very slowly by normal biological fluids. A testing program with enzymes and membranes has been initiated. Studies are also in progress on the synthesis of polypeptide hormones containing 2-fluorohistidine. (K. L. Kirk, W. Nagai, and L. A. Cohen).

Fluoro Analogs of Polynucleotide Precursors

Studies on the synthesis of fluoroimidazoles have been extended to include analogs of purine precursors. Thus, 4-fluoroimidazole-5-carboxamide and 2-fluoro-4-aminoimidazole-5-carboxamide (as well as the 2,4-diamino analog) have been prepared. These species are now being converted into their respective ribotides and phosphoribotides, which will be examined as substrates or inhibitors of purinic acid biosynthesis, and as elements of incorporation into DNA or RNA. (K. L. Kirk, W. Nagai, and L. A. Cohen).

2-Aminohistidine as an Arginine Analog

Aryldiazonium coupling to N-acetylhistidine methyl ester produces a mixture of 2-, 4-, and 2,4-bis-arylazo derivatives. Painstaking chromatography has permitted to isolation of the pure 2-arylazo derivative. Reduction and hydrolysis of this compound provides 2-amino-L-histidine. N-acetyl esters, amides, and peptide derivatives of this amino acid are cleaved by trypsin at the carboxyl group. Since this amino acid may be viewed as a cyclic analog of arginine, the implication exists that arginine must coil into a *pseudo*-cyclic structure to fit the active site of the enzyme. Studies are in progress to effect the chemical conversion of histidine in polypeptides into 2-aminohistidine, in order to provide a new site for selective proteolytic cleavage of the peptide chain. (W. Nagai, K. L. Kirk, and L. A. Cohen).

Labeling of Biological Materials with Carrier-free ^{125}Iodine

Because of numerous difficulties with the techniques currently used for generation of iodine and iodination in radioimmune assay methods and iodination of hormones, modifications have been introduced. In the present method, ^{125}iodide is oxidized to iodine monochloride with excess Chloramine T, in the presence of the material to be iodinated. Since excess Chloramine T destroys peptides and other materials by chlorination and oxidation, much of the material to be assayed can be lost. In the new method, the iodide is oxidized with Chloramine T, but the iodine monochloride is extracted from the mixture with benzene, and the extract is added to the biological substrate in a separate container. Alternatively, the iodine monochloride is added to silver succinimide, to generate the solid, stable, and nonvolatile N-iodosuccinimide. This material can be used directly for similar iodinations, or can be stored as a stock solution or as a solid for subsequent use. The entire procedure is performed in a closed system, using only hypodermic syringes for transfer, the latter being introduced through a rubber septum. Thus, the danger inherent in the use of volatile radioiodine compounds is eliminated, the integrity of the substrate is retained, and a much more specific iodination is achieved. (L. A. Cohen).

Specific Radioiodine Labeling of Membrane Proteins

An insoluble polymer containing N-iodosuccinimide units has been developed to iodinate selectively highly exposed tyrosine or histidine residues in soluble proteins. This polymer fails to iodinate insoluble protein, proving that the iodinating agent is not a small molecule which is liberated into the aqueous medium. More recently, a water-soluble polymer has been developed which contains the same N-iodosuccinimide units. This material can iodinate protein membranes (such as erythrocyte ghosts) over a wide pH range. Finally, a technique has been developed to introduce carrier-free ^{125}iodine into the polymer. It is, therefore, possible to radioiodinate substrates in solution with an insoluble polymer reagent, or to iodinate an insoluble substrate with a soluble polymer reagent. In either case, the simplicity of separation after reaction is obvious. Plans

are under way to make these polymer reagents available to the biochemical and medical communities for specific application.
(R. Jerussi and L. A. Cohen).

Affinity Columns for Receptor Proteins

By means of diazonium coupling into the phenolic ring, phenol- and catecholamines have been bound to insoluble sepharose supports. Since catecholamines are very sensitive to oxidation in basic media, the achievement of diazonium coupling without adrenochrome formation posed a serious obstacle. However, this problem was eliminated with the discovery that diazonium coupling can be achieved at pH values as low as 5, but only when the diazonium salt is part of an insoluble macromolecule. In this respect, the reaction mimics the enzyme-substrate complex, in that the pH required in effecting a difficult reaction is reduced by the intramolecular effect of molecule-macromolecule association. When Metaraminol (a known inhibitor of norepinephrine binding) is bound to sepharose, a suspension of the polymer inhibits uptake of norepinephrine by some tissue slices. Application of this technique for isolation of receptor protein is in progress. (I. Hanbauer and L. A. Cohen).

Stereopopulation Control as an Explanation for Enzyme Catalysis

As studies continue in the field of stereopopulation control, it becomes increasingly clear that the contribution of substrate binding to the overall catalytic function of enzymes has been grossly underestimated. On the basis of the rate enhancement factors already obtained in various model systems, we believe that the deformation and rotational freezing of the substrate may, in fact, be more important than the catalytic activities of active-site groups. Thus, studies have shown that a slight conformational twist of a saturated pyran ring (as in hexoses) is sufficient not only to alter reaction rate by a factor of 3000, but to change completely the mechanism of a reaction, without changing the nature of the product. Other studies with models for oxygen and nitrogen lyases have shown that, whereas buffer catalysis contributes powerfully to reaction rate, such catalysis is totally lost in the absence of severe conformational freezing. The validity of these concepts can be tested by design and synthesis of substrate analogs (and of drugs) which are subject to severe conformational freezing. Efforts in these directions have been initiated. (L. A. Cohen).

Stereopopulation Control in the Formation of High Energy Bonds

Alkylated analogs of homophthalic acid have been synthesized in which the carboxyl groups are frozen in very close proximity to one another. These acids undergo cyclization to their anhydrides spontaneously or with mild acid catalysis. Under the same conditions, any homophthalic acid in which there exists a flexibility of conformation shows no tendency to form an anhydride. It is estimated that the entropic effect of conformational freezing favors the anhydride at equilibrium to the extent of 10^6 and increases the rate constant for cyclization by at least 10^9. These studies demonstrate quite clearly that the binding of a substrate to an enzyme is

itself a powerful activating process and can, in principle, be sufficient to create a covalent bond, and even a high energy bond, without the requirement of ATP or other external source of free energy. Similar studies with derivatives of imidazolepropionic and imidazolebutyric acids have continued and lead to comparable conclusions.
(P. S. Hillery, Y. Kikugawa, and L. A. Cohen).

SECTION ON CARBOHYDRATES

2-Acetamido-2-deoxyaldonic Acids

Three chemical processes have been developed which have permitted the isolation of the first-known 2-acetamido-2-deoxyhexonic acids--a new class of substance representing the aldonic acids related to the biochemically important *N*-acetylaminosugars. These structures are comparatively stable to alkali and thus 2-acetamido-2-deoxy-D-mannonic acid can be converted into its hitherto unknown dicyclohexylammonium salt through the action of aqueous dicyclohexylamine. In contrast to these free acids, the corresponding 2-acetamido-2-deoxy-D-mannono-1,4-lactone affords a mixture of the dicyclohexylammonium salts of 2-acetamido-2-deoxy-D-mannonic acid and 2-acetamido-2-deoxy-D-gluconic acid when treated with aqueous dicyclohexylamine. With nonaqueous dicyclohexylamine, the lactone undergoes a β elimination to give 2-acetamido-2,3-dideoxy-D-*erythro*-hex-2-enono-1,4-lactone. It is now clearly evident that the 2-acetamido-2-deoxyaldonic acids and their derivatives have many interesting properties which are not shown by the much better known aldonic acids having a hydroxyl group at carbon 2.
(E. Zissis, H. W. Diehl, and H. G. Fletcher, Jr.).

Unsaturated Aminosugars

Earlier studies have shown that various nucleophiles react with 3,4,6-tri-*O*-acetyl-2-(*N*-acetylacetamido)-1,5-anhydro-2-deoxy-D-hex-1-enitols when heated in the presence of an acid catalyst to give 4,6-di-*O*-acetyl-2-(*N*-acetylacetamido)-2,3-dideoxy-α-D-hex-2-enopyranose derivatives. The overall transformation appeared to arise from a nucleophilic attack on carbon 1, accompanied by loss of the acetoxy group at C-3 and allylic shift of the double bond. Subsequent studies have now shown that this view of the mechanism represents an oversimplification. It now appears that the starting product undergoes a preliminary series of rearrangements, the acetoxy group at C-3 migrating with allylic shift to the β side of C-1, followed by anomerization to the thermodynamically more stable 1,4,6-tri-*O*-acetyl-2-(*N*-acetylacetamido)-2,3-dideoxy-α-D-hex-2-enopyranose. The C-1 acetoxy group in this product is then displaced by the nucleophile to yield the observed final product.

Two pairs of anomers, the 1,4,6-tri-*O*-acetyl-2-(*N*-acetylacetamido)-2,3-dideoxy-D-hex-2-enopyranoses of the *erythro* and *threo* series have been prepared and characterized. Nuclear magnetic resonance studies of these structures suggest that the α-D anomers exist in the H_{5}° conformation while the β-D anomers assume a conformation which is closer to H_{5}° than $H_{\circ}^{5}$.

Studies of the behavior of a variety of 1,4,6-tri-*O*-acyl-2-(*N*-acetylacetamido)-[and acetamido]-2,3-dideoxyaldohex-2-enoses with hydrogen in the presence of a palladium catalyst have revealed a remarkable specificity. While β-D anomers are not attacked, all α-D anomers (with one exception) undergo allylic hydrogenolysis at C-1. An interpretation of this unusual situation has been proposed.

A simple method for the conversion of N-acetylacetamido derivatives to acetamido compounds (i.e. a mono-N-deacetylation has been devised. (N. Pravdic, B. Zidovec, I. Franjic, and H. G. Fletcher, Jr.).

The Isopropylidenation of Certain Aldoses

When treated at room temperature in N,N-dimethylformamide solution and in the presence of a trace of p-toluenesulfonic acid with a small molar excess of 2,2-dimethoxypropane, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose, 2-acetamido-2-deoxy-D-galactose and D-glucose gave the corresponding 4,6-O-isopropylidenealdopyranoses. All of these derivatives are new and are not given by any of the older isopropylidenating reagents. D-Mannose, on the other hand, affords the known 2,3:5,6-di-O-isopropylidene-D-mannofuranose. The yields from these reactions are high and the structures of the new products have been elucidated unequivocally.

When the reagent mixture is used at 80° the results are almost totally different. 2-Acetamido-2-deoxy-D-mannose gives a mixture from which methyl 2-acetamido-2-deoxy-2,3-N,O-isopropylidene-5,6-O-isopropylidene-α-D-mannofuranoside, 2-acetamido-2-deoxy-2,3-N,O-isopropylidene-5,6-O-isopropylidene-D-mannofuranose, and methyl 2-acetamido-2-deoxy-5,6-O-isopropylidene-α-D-mannofuranoside were isolated. 2-Acetamido-2-deoxy-D-galactose gave compounds which were identified as methyl 2-acetamido-2-deoxy-5,6-O-isopropylidene-β-D-galactofuranoside and methyl 2-acetamido-2-deoxy-4,6-O--isopropylidene-β-D-galactopyranoside. 2-Acetamido-2-deoxy-D-glucose yielded methyl 2-acetamido-2-deoxy-5,6-O-isopropylidene-β-D-glucofuranoside and methyl 2-acetamido-2-deoxy-4,6-O-isopropylidene-β-D-glucopyranoside. All of these products represent hitherto unknown structures. Under these more vigorous conditions D-mannose gave the known 2,3:5,6-di-O-isopropylidene-D-mannofuranose although in notably higher yield than had been found with the milder conditions.

These new carbohydrate derivatives offer great potential for the synthesis of hitherto inaccessible carbohydrate structures. (A. Hasegawa and H. G. Fletcher, Jr.).

Immunochemistry

Seven homogeneous IgA myeloma immunoglobulins have been isolated from mice. These all specifically bind the same antigen, a β 1→6 polygalactose. The binding has been studied (Ka) by quantitative measurement of the increase of fluorescence of the antibody upon combination with ligand. This change in fluorescence has been traced to a tryptophan residue in the active site. The above antibodies, although having the same specificity, are all idiotypically different. They have been sequence-analyzed up to residue 50 (with S. Rudikoff and M. Potter). One IgA

(Fab fragment) has crystallized sufficiently well for X-ray crystallographic investigation, which is about to begin (with D. Segal, E. Padlan, M. Potter, and S. Rudikoff). Radioactively labeled ligand has been prepared for binding constant-studies on the crystalline material. Affinity labeling of the IgA antigalactans will be attempted by reaction with epoxy galactosides, and by reaction with diazotized 6-O-glycyl derivatives of galactosides under the influence of light and Cu++ so as to insert carbenes into the protein chain. The necessary intermediates have been prepared. Through inhibition studies it has been shown that beef lung galactan, which also reacts with the above IgA's, has a branched structure and not an essentially linear structure as was originally proposed. (C. Glaudemans and M. Jolley).

SECTION ON MEDICINAL CHEMISTRY

Theoretical Analyses in Medicinal Chemistry

Molecular orbital studies of biogenic amines and Hansch analysis of catechol *O*-methyltransferase substrates have been carried out to provide information on the mechanism of drug action, receptor-drug interaction and more rational approaches to drug design. Minimum-energy conformations, e.g., of 6-hydroxydopamine and other polyhydroxyphenethyl amines as well as dopamine and norepinephrine have shown that these compounds can exist in their gauche form with certain heteroatoms at a specific (*ca.* 6 Å) distance. The molecular requirements for long-term reduction in cardiac uptake of norepinephrine have been extended using this criterion (heteroatom-interatomic distance) (A. E. Jacobson).

Studies in the Morphine Series

The Wittig reaction on nordihydrocodeinone utilizing methylsulfinyl carbanion dimethyl sulfoxide has given two chromatographically separable products of mass number 297 (crystalline) and 283 (sirupy). The latter is consistent with replacement of the carbonyl oxygen by CH_2 (normal Wittig), the former with introduction of an additional CH_2 species, perhaps at the NH function (as indicated by nmr) (L. J. Sargent).

Reaction of morphine with dimethylchloroformiminium chloride has given not the expected 3,6-diformylmorphine but a mixture which includes 6-formylmorphine and a so-called "α-chloromorphide" first described in 1934. This has been proved to be a 6-isochloro compound (nmr analysis) (A. E. Jacobson).

Agonist-Antagonist Evaluation

Eighty-six new compounds (45 from the pharmaceutical industry, the remainder from universities and the synthesis program of this Section) were tested for analgesic activity by the hot-plate procedure. Of these, 33 were considered sufficiently interesting for complete assay. Forty-seven of the above were also tested by the Nilsen procedure, more predictive for compounds having dual (agonist-antagonist) action. Several of these compounds were further evaluated in monkeys at the University of Michigan for physical dependence and antagonistic property. Several promising candidates, long-acting and orally effective, have been uncovered. Efforts to develop a simple method for narcotic antagonistic effect in small animals have failed thus far (L. Atwell, E. L. May, E. McNeal).

Research Toward Improved Synthetic Methods

Because of the medical potential of certain phenylmorphans, 3-benzazocines and 9-hydroxy-6,7-benzomorphans, improved methods of synthesis are warranted. A 9-step sequence from 2-carbethoxymethyl-2-m-methoxyphenylcyclohexanone involving two "Sarett" oxidations has given the desired 5-m-methoxyphenyl-2-methyl morphan. Thus far, cyclization of N-cyanomethyl-N-methyl-3-m-methoxyphenylbutylamine (and derivatives) and N-p-methoxyphenethyl-N-tosyl-3-aminopropionic acid, both prepared in three, good-yield steps have failed, decarbonylation generally taking place with polyphosphoric acid (M. E. Rogers).

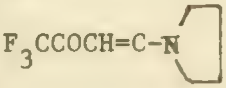
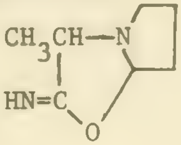
Schmidt reaction of 2,3-(3-methoxybenzo-1,8-dimethyl-8-hydroxybicyclo-[3.2.1]-oct-2-en-6-one (prepared from 7-methoxy-β--tetralon in several steps) gave less than 40% of the corresponding-3-oxo-benzomorphan. 1,2-Dimethyl-7,8-(3-methoxybenzo)bicyclo-[3.3.0]-octa-2,7-dien-4-one (from acid-catalyzed rearrangement) was the major product. Similar reaction of the corresponding acetyl derivative under a variety of conditions gave similar results (R. S. Wilson).

Mechanism of Action of Δ^1- and Δ^6-Tetrahydrocannabinol

It has been stated by some investigators that the biological activity of Δ^1- and Δ^6-tetrahydrocannabinol (THC), the active principles of marihuana, is due to predominant (active) metabolites, 7-hydroxy-THC's. Of interest, therefore, would be 7-nor-Δ^1 and Δ^6-THC'S not capable of such metabolism. Efforts to synthesize these compounds have progressed to (±)-6aβ,7,10,10aα-tetrahydro-1-hydroxy-6,6-dimethyl-3-pentyl-6H-dibenzo[b,d]pyran-9(8H)-one (I), borohydride reduction to the corresponding diol (II) and acid catalyzed dehydration of II to give, apparently, 7-nor-Δ^6-THC. Conversion of II to the chloride was effected in 40% yield in two ways. Appropriate dehydrohalogenation should give 7-nor-Δ^1-THC.

Diol II (with the Lucas reagent) surprisingly, underwent selective acetylation of the alcoholic hydroxyl, and tetrahydropyranylation of I resulted in C-alkylation ortho to the phenolic hydroxyl, perhaps a novel reaction of vinyl phenols (R. S. Wilson).

Synthetic Methods for Alkaloids

Attempted hydration of the iminium salt of 2-(1-pyrrolidinyl)-propionitrile (derived from the N-oxide via a modified Polonovski transformation has yielded products which are solvent dependent. Trifluoroacetic anhydride give  in ether and  in benzene.

When a more nucleophilic acid was present, the normal (N-acyl) Polonovski product was isolated. Attempts to replace the α-hydrogen with hydroxyl yielded nothing useful (E. M. Fry).

Synthesis of 9-Methyl-6,7-Benzomorphans

Starting from phenylacetonitrile, an 11-step route to 9-methylbenzomorphans has been developed *via* a diastereoisomer of 4-dimethylamino-3-methyl-1,2,3,4-tetrahydro-2(1H) naphthalenone, the key step being the Reformatsky reaction of 5-dimethylamino-3-phenyl-2-pentanone and ethyl-bromoacetate. The second possible diastereoisomer of the intermediate naphthalenone, on cyclization with NH_4OH, did not give the expected benzomorphan skeleton but 4-dimethylamino-3-methyl-1-naphthol. This scheme should pave the way for synthesis of the long-sought 2,9-dimethyl-2′-hydroxy-6,7-benzomorphan. This would be a second example of a non-quaternary carbon benzomorphan, the 9-desmethyl homolog of which is an agonist-antagonist presently under pharmacologic study (T. Oh-ishi).

Some Typical and Atypical Narcotic Antagonists and Agonist-Antagonists

The *levo*-isomer of α-2-allyl-5,9-dimethyl-2′-hydroxy-6,7-benzomorphan has been prepared and found to be three times as potent as nalorphine with a similar duration of action. The (+)-isomer is devoid of properties of antagonism but produces non-narcotic, CNS depressant effects with marked ataxia, effects not reversed by nalorphine.

Replacement of the methyl group of 5-*m*-hydroxyphenyl-2-methylmorphan and its (-)-isomer (absolute configuration determined by x-ray crystallography, Dr. Todd Cochran, University of Duquesne), strong analgesics with an equatorial *m*-phenolic group attached to the quaternary carbon, by propyl or cyclopropylmethyl conferred only weak properties of antagonism. This is in contrast to experience in the benzomorphan, morphinan and morphine series which have a fixed axial *m*-phenolic quaternary-carbon substituent. Allyl for methyl simply *reduces agonistic* activity in the phenylmorphans.

With the heroin-like ketobemidone (also containing an equatorial, m-phenolic radical), allyl, propyl and cyclopropylmethyl in place of N-methyl also made for weaker analgesic (no antagonistic) properties. However, N-amylnorketobemidone is four times more potent than ketobemidone as an analgesic, shows no physical dependence properties in monkeys and displays some atypical antagonistic effects. N-hexyl and N-heptylnorketobemidones are weak, long-acting, nalorphine-like antagonists with analgesic activity between morphine and pethidine.

These results indicate that a fixed, axially oriented *m*-phenolic group attached to the quaternary carbon is a structural requisite for strong narcotic, antagonistic behavior. Of further interest is that higher alkyl groups (at least in the 4-phenylpiperidine series) are potentially valuable N-substituents and can confer antagonist behavior (T. Oh-ishi, H. Ong and E. L. May).

Several O-acyl derivatives of (±)-2,5-dimethyl-2´-hydroxy-6,7-benzomorphan, strong analgesics, display also properties of antagonism in morphine-dependent monkeys and will not suppress abstinence at any sub-toxic dose (J. H. Ager).

SECTION ON METABOLITES (in part with Section on Pharmacodynamics)

Effects of Batrachotoxin on Electroplax Na$^+$-K$^+$-ATPase and Levels of ATP in Rat Muscle

Batrachotoxin (BTX) in low concentrations (20 nM) depolarizes electrically excitable membranes. At these levels, BTX does not inhibit Na$^+$-K$^+$-ATPase. At much higehr concentrations (60 μM) BTX partially inhibits Na$^+$-K$^+$-ATPase from electroplax of *Electrophorus electricus*. In contrast to inhibition by cardiac glycosides, the inhibition of Na$^+$-K$^+$-ATPase by batrachotoxin is not antagonized by KCl. BTX had no effect on ATP levels in stimulated nerve muscle preparations at the time when sustained contracture was initiated by the drug. Phosphocreatine levels were decreased and levels of glucose-6-phosphate and 6-phosphogluconate were increased, while levels of fructose-1,6-diphosphate and α-ketoglutarate were unchanged. It is concluded that the inhibition of Na$^+$-K$^+$-ATPase or lowering of ATP levels by BTX is not significantly involved in the membrane dopolarization produced by the toxin (J. Daly, E. X. Albuquerque, F. C. Kauffman and F. Oesch).

The Pharmacology of Batrachotoxin. A Comparative Study of Membrane Properties and the Effect of Batrachotoxin on Sartorius Muscles of the Frogs Phyllobates aurotaenia and Rana Pipiens

Membrane properties and the effects of batrachotoxin (BTX) and tetrodotoxin were studied on sartorius musches of *Rana pipiens* and of *Phyllobates aurotaenia*, the frog which produces BTX. There was no significant difference in the resting membrane potential amplitude and time course of miniature end-plate potentials to the quantum content recorded in sartorius muscles of the two frogs. The frequency of spontaneous transmitter release was, however, higher in the sartorius muscles of *P. aurotaenia* than in *R. pipiens*. Both the time constant and specific resistance of a unit area of membrane of the sartorius muscles of *P. aurotaenia* were significantly lower ($P < .01$) than those of *R. pipiens* muscle fibers. The intracellularly recorded action potential of sartorius muscles from both frogs was blocked by removal of the external sodium or by exposure to tetrodotoxin. BTX (1.2×10^{-6} M) had no effect on membrane potential, miniature end-plate potential amplitude and frequency, spike generation or twitch of the nerve-muscle preparations of *P. aurotaenia*. When sartorius muscles of *R. pipiens* were stimulated at a frequency of 0.2 pulse/sec and exposed to BTX (4.0×10^{-8} M), the indirectly elicited muscle twitch was blocked and all surface fibers were depolarized within 13 minutes; simultaneously, there was a transient muscle contracture lasting seven to eight minutes. Veratridine (7.5×10^{-6} M) depolarized the surface fibers of stimulated sartorius muscles from *P. aurotaenia* by 20 mV after one hour but those of *R. pipiens* were depolarized by 95 mV within 15 minutes. BTX-depolarized sartorius muscles of *R. pipiens* were insensitive to isotonic

KCl, whereas caffeine (20 mM) induced a normal contracture. Electron microscopic observations of BTX-treated sartorius muscles from R. pipiens revealed damage to only the presynaptic elements, whereas the nerve and muscles of P. aurotaenia were unaffected. It is concluded that: 1) in sartorius muscle fibers of P. aurotaenia, the early inward current during an action potential is carried by sodium ions; 2) the electrogenic membranes of the sartorius muscles of P. aurotaenia are insensitive to the sodium-dependent depolarizing action of BTX observed in R. pipiens and in mammalian nerve and muscle membranes, but they remain sensitive to the blocking action of tetrodotoxin and 3) the site of action of BTX may be absent in nerve and muscle membranes of P. aurotaenia (E. X. Albuquerque, J. E. Warnick, F. M. Sansone and J. W. Daly).

Acetylcholine Receptor and Ion Conductance Modulator Sites at the Murine Neuromuscular Junction: Evidence from Specific Toxin Reactions

The perhydroderivative of histrionicotoxin reversibly blocks the excitatory ionic transduction system in the synaptic and sarcolemmal membranes of mammalian skeletal muscle cells. The efficacy of perhydrohistrionicotoxin as an antagoist at the post-synaptic membrane is increased by the transient presence of acetylcholine in the endplate of innervated muscles and at extra-junctional receptors in denervated muscles. α-Bungarotoxin and [^{3}H]monoacetyl-α-bungarotoxin block the endplate acetylcholine receptors, each binding to the same extent. The effect of bungarotoxin is partially reversible. These electrophysiological results, together with the effects of perhydrohistrionicotoxin and/or d-tubocurarine on the binding of [^{3}H]monoacetyl-α-bungarotoxin at endplates of murine diaphragm muscle and on the bungarotoxin-elicited irreversible blockade of neuromuscular transmission, suggest that at least two types of sites participate in the synaptic excitation by acetylcholine. One site, competitively blocked by bungarotoxin and by curare, is presumably the acetylcholine receptor. Binding of bungarotoxin at this site is responsible for an irreversible blockade of neuromuscular transmission. The second site, competitively blocked by bungarotoxin and perhydrohistrionicotoxin, is proposed to be part of the cholinergic ion conductance modulator. Binding of bungarotoxin to this site does not result in an irreversible blockade (E. X. Albuquerque, E. A. Barnard, T. H. Chiu, A. J. Lapa, J. O. Dolly, S. Jansson, J. Daly and B. Witkop).

Congeners of Histrionicotoxin

There are now 9 congeners of histrionicotoxin known. Some have been isolated (T. Tokuyama) in crystalline form. They all have inhibitory activity relative to acetylcholine, if histrionicotoxin itself is given a value of 1, as follows: Dihydroisohistrionicotoxin (one vinylacetylene, one allene side chain) 4; natural neodihydrohistrionicotoxin (one vinylacetylene, one diene side chain) 2; synthetic dihydrohistrionicotoxin (diene in the other side chain) 2; allenic tetrahydrohistrionicotoxin (allene in the 5-carbon, diene in the 4-carbon chain) 2; tetrahydrohistrionicotoxin (diene in both side chains) 2; octahydrohistrionicotoxin (terminal double bonds in both side chains) 20-40; synthetic (J. Daly) dodecahydrohistrionicotoxin (fully saturated side chains) 2. There is also a histrionicotoxin with a 3 carbon allyl side chain (G. Brown). There are other parameters by which to evaluate the order of activity which are now in the process of determination in the Laboratory of E. X. Albuquerque.

Synthesis of Perhydrohistrionicotoxin

While the synthesis of histrionicotoxin in the laboratory of E. J. Corey at Harvard University is proceeding very slowly, there are several synthetic approaches to perhydrohistrionicotoxin underway, with the most concerted effort going on in the laboratory of Professor O. Jeger at the ETH, Zurich, under the direction of H. Wehrli who so successfully concluded the 37-step synthesis of batrachotoxin.

Synthesis of Cholinergic Agonists

As a continuation of his synthetic approach to the *cis*-decahydroquinoline alkaloid *pumiliotoxin C*, isolated from the frog *Dendrobates pumilio*, Dr. Wolfgang Kissing, Guest Worker from the Laboratory of Professor G. Habermehl, Technische Hochschule, Darmstadt (Germany), is preparing aminoalcohols derived from hydrogenated quinolines to be evaluated for their cholinergic activity.

Conformations of the Li-Antamanide Complex and Na-[Phe^4, Val^6]Antamanide Complex in the Crystalline State

Antamanide, a cyclic decapeptide isolated from the poisonous mushroom *Amanita phalloides*, complexes with Li$^+$ and Na$^+$ but not with K$^+$. The selectivity of complexation makes it an important model for the study of ion transport across cell membranes. The conformations of the lithium antamanide complex and the sodium [Phe^4, Val^6] antamanide complex have been established by x-ray diffraction analyses of single crystals. The two compounds are isostructural, but not isomorphous. The complexes are folded into a globular shape with an approximate two-fold axis. Two of the peptide linkages are in the *cis* conformation, Pro^2-Pro^3 and Pro^7-Pro. There are only two intramolecular hydrogen bonds. Four C=O groups have their O atoms directed inward to form four Li-O or Na-O ligands. The fifth ligand to the metal ion is provided by a solvent molecule. The conformation found in the crystalline state is different from any of the conformations proposed for the sodium antamanide complex in solution on the basis of NMR data (I. L. Karle, J. Karle, Th. Wieland, W. Burgemeister, H. Faulstich and B. Witkop).

Effect of Pyrimidine and Ribose Modifications on the Antiviral Activity of Synthetic Polynucleotides

Homo- and copolynucleotides derived from 3-methyluridine, 5,6-dihydrouridine and 2'-azido-2'-deoxyuridine (dUz) were prepared and evaluated for antiviral activity either as single strands or as double strands when complexed with complementary homopolynucleotides. The synthetic materials were not as active as $(A)_n.(U)_n$ or $(I)_n.(C)_n$ in inducing the interferon system. The antiviral activity of $(A)_n.(dUz)_n$ was not affected by treatment with high concentrations of pancreatic RNase A, whereas this activity was totally abolished when $(A)_n.(dUz)_n$ was digested with human serum (P. F. Torrence, J. A. Waters, C. E. Buckler and B. Witkop).

Synthesis and Characterization of Potential Interferon Inducers: Poly(2'-Azido-2'-Deoxyuridylic) Acid

2'-Azido-2'-deoxyuridine (I) was converted in a series of steps to 2'-azido-3'-acetyl-2'-deoxyuridine (IV) which was phosphorylated with 2-cyanoethyl-phosphate to give 2'-azido-2'-deoxyuridine 5'-monophosphate (V) from which the 5'-diphosphate (VI) was prepared by the morpholidate procedure. Polynucleotide phosphorylase polymerized VI in the presence of Mg^{++} or Mn^{++} to poly(2'-azido-2'-deoxyuridylic) acid [poly(dUz)] which was completely resistant to degradation by 0.3 N KOH and pancreatic ribonuclease A, as well as DNase I. Circular dichroism studies indicate that at 2.5°, poly(dUz) assumes a secondary structure similar to that of poly(U) (most likely a single hairpin). Ultraviolet and CD-temperature profiles in a number of buffer systems indicate that this structure has a thermal stability which is somewhat greater than poly(U). This finding indicates that neither a 2'-OH group nor a 2'-oxygen atom is necessary for the formation of ordered structure in poly(U).

The construction of mixing curves at the isochromic wavelengths for this system revealed that poly(dUz) can form both double- and triple-stranded complexes with poly(A), *i.e.*, poly(dUz) . poly(A) and 2 poly(dUz). poly(A). Study of the course of the thermal dissociation of these complexes undergo monophasic transitions directly to constituent homopolymers (3 → 1 and 2 → 1, respectively). Unlike the previously reported A-U system and the A-rT system, no evidence for strandwise dissociation (3 → 2) or rearrangement (2 → 3) could be found. This unusual behavior makes the assignment of the above transitions tentative until further evidence can be obtained. If the assignment of the above transitions (3 → 1 and 2 → 1) is correct, then the introduction of the azido group into the C2' position of poly(U) does nto have a significant effect on the stabilities of both the triple- and double-stranded complexes when compared with the corresponding complexes in the poly(A)-poly(U) system (P. F. Torrence, A. M. Bobst, J. A. Waters and B. Witkop).

Synthesis of Potential Antitumor and Antivirus Agents

One-step introductions of substituents into the 5- or 6-positions of (protected) nucleosides continue and J. A. Waters is scaling up the photochemical introduction of the cyano group into the 6-position of uridine for evaluation.

Transient Cyclohexadienones Through Novel Reversal Reactions from Cage Photodimers derived from N-Chloroacetyltyramines

In a collaborative effort with O. Yonemitsu, University of Hokkaido, it was shown that the novel dimeric cage compounds obtained by irradiation of substituted N-chloroacetyltyramines undergo a novel type of cyclic reversion reaction by digestion with trifluoroacetic acid, whereby the type and location of alkyl substituents determined the length and temperature of acid digestion. In addition, N-ethylmaleimide trapped transient cyclohexadienone intermediates as Diels-Alder adducts both starting from N-chloroacetyltyramines and irradiation, as well as by pyrolysis of the photodimer in the presence of dienophile (T. Iwakuma, O. Yonemitsu, N. Kanamaru, K. Kimura and B. Witkop).

Approaches to Analogs of Anhydrogliotoxin

The addition of α-halo-α-aminoacyl chlorides to ethyl indolenine-2-carboxylates followed by reaction with sulfur nucleophiles and a final ring-closure provides a convenient and new synthetic scheme to analogs of gliotoxin, the simplest of the natural products containing the epidithiodiketopiperazine system. Illustrative of this approach, adducts of ethyl 3,3-dimethylindolenine-2-carboxylate with acid chlorides, α-halo acid chlorides and N-trifluoroacetyl-α,α-dichlorosarcosyl chloride have been studied. The last adduct when treated with a sulfide-polysulfide mixture gave a monosulfide (30% yield) but no disulfide. Reduction of the monosulfide with $NaCNBH_3$ proceeded stereoselectively to afford mainly the seco-gliotoxin analog in addition to the other diastereoisomer. Cyclization of this mixture presumably led to the strained epimonothio-diketopiperazine which easily opened to isomeric lactams (H. C. J. Ottenheym, T. F. Spande and B. Witkop).

SECTION ON MICROANALYTICAL SERVICES AND INSTRUMENTATION

Service Function and Instrumentation

Approximately 30 elements and five functional groups are determined routinely by the microanalytical laboratory. The total analyses were 13,256, a 10.5% increase over the previous year. Routine instrumental service continues at a high level in the areas of IR, NMR, and mass spectrometry. Several thousand samples were run for investigators in the laboratory by specialists in each area (D. F. Johnson).

The Study of Steroid Transformations by Tetrahymena Pyriformis

Previous studies have demonstrated that a variety of steroids of mammalian origin as well as steroid derivatives such as dialkylaminoethoxy steroids inhibit the growth of the protozoa, T. pyriformis, and that this growth inhibition is reversed by the addition of cholesterol. The mode of action of these growth inhibatory steroids is not definitely known, but inhibition of NADH oxidation and RNA synthesis, and interference with respiration and formation of lipids have been demonstrated. T. pyriformis grown without added steroids produces a pentacyclic triterpnoid alcohol, tetrahymenol as its major unsaponifiable product by a direct, non-oxidative, proton initiated cyclization of squalene. When steroids such as dehydroepiandrosterone are added to cultures, an accumulation of squalene occurs with a corresponding decrease in growth rate of the organism. Since the focus of attention has been on the action of steroids on tetrahymena, the present studies are designed to study the effect of the protozoa on the steroid molecule. Substrates incubated to date include androstenedione, progesterone, 17α-OH-progesterone, testosterone, and corticosterone. Preliminary data from column and thin-layer chromatography indicates dehydrogenation and hydroxylation as possible transformations. Additional substrates are being used and products isolated to help elucidate the role of steroid metabolism in tetrahymena (N. Lamontagne and D. F. Johnson).

Molecular Orbital Studies of Catechol-O-Methyltransferase and Biological Amines

Modern statistical and computational methods were used to study structure-activity relationships as well as chemical behavior of some biologically important systems and compounds. The Hansch approach and program was used for the regression analysis of the physico-chemical and molecular orbital data assembled. A correlation of the ratio (meta:para) of O-methylated neutral side-chain catechol derivatives with the steric effect of the substituent, as well as the transport factor, was found. Maximum reaction velocity for the substrate-enzyme reaction could be correlated with the steric and electronic parameters of the substrates.

Minimum energy conformations of 6-hydroxydopamine and other polyhydroxyphenethylamines, as well as dopamine and norepinephrine, were determined. It was found that all of these compounds can exist in their gauche form with certain heteroatoms at a specific distance of about 6Å. The molecular requirements for long-term reduction in cardiac uptake of norepinephrine have been suggested using this criterion of heteroatom interatomic distance.

Using the molecular orbital (CNDO/2) method on the twisted cyclohexadiene ring system of trans-diaxial-chorismic acid, minimum energy conformations, dipole moments, and charge distributions were calculated. These data contribute to a better understanding of the chemical and spectral behavior of these important chemical intermediates (R. Katz, A. E. Jacobson, S. R. Heller, and R. J. Feldman).

The Study of the Photochemical Reactions of Certain Derivatives of Indole and Related Heterocyclic and Carbocyclic Compounds

In a continuing study of the photocyclodehydrohalogenation reaction produced by uv irradiation of N-chloroacetyltryptamine and related compounds, various products have been isolated and characterized. The product of the photodehydrohalogenation of N-chloroacetyltryptamine is the tricyclic lactam, 6-oxo-3,4,6,7-tetrahydro-1H,5H-azocin (4,5,6-c,d) indole, which results from cyclization in the 4-position of the indole moiety. Chemical and NMR studies have provided conclusive evidence for the assigned course of the cyclization. This tricyclic amine is also of interest because of its potential physiological activity.

Studies in progress of the photolysis products of N-chloroacetyl-3-(3′-aminopropyl)indole and N-chloroacetyl-3-(4′-aminobutyl)indole are underway. It is expected that NMR spectroscopy of the amines derived from them will provide final evidence needed to define their structures. These amines will also be of pharmacological interest (C. Foltz).

The Study of the Constituents of Solanum Xanthocarpum

A highly toxic component toward the housefly larvae was found in the extracts of S. Xanthocarpum in previous investigations in this laboratory. A number of steroidal compounds were identified from this extract, among which were solasonine, solamargine, and solasodine. Although the three alkaloids showed some larvacaedal activity, the most toxic substance was not found. Subsequent investigation has revealed that solamargine and solasonine, in mixture, have a synergistic, highly toxic effect. Studies are continuing to to establish the optimum ratio for this activity (G. Kusano, R. Katz, D. F. Johnson, and Y. Sato).

Metabolism of ^{14}C-Labelled Steroids by Adrenals from Rats with a Mammotropic Pituitary Tumor

Earlier studies from this laboratory demonstrated abnormalities in the biogenesis of adrenal steroids by adrenal glands from rats with a mammotropic pituitary tumor (MtT). Reduced conversion of progesterone and qualitative differences of metabolic products was found. Most recent results show that the conversion of pregnenolone to progesterone is also markedly reduced and isolation of metabolites indicates a by-pass biosynthetic pathway than that of normal rat adrenals. Studies are continuing to elucidate the pathway involved and identify the metabolites formed (D. F. Johnson and N. Lamontagne).

Structure Determination of Tomatillidine

In the course of investigations of dihydrotomatillidine, it was found that an unexpected isomerization occurred during chromatography of intermediate chemical steps. Spectroscopic data of these compounds raised questions concerning the accepted structure of tomatillidine. Subsequent synthetic and NMR studies indicate a new assignment for the structure of tomatillidine. These findings can lead to a better understanding of the biosynthetic role of this alkaloid in plant (G. Kusano, D. F. Johnson, and H. Yeh).

^{31}P-^{1}H Nuclear Magnetic Resonance Studies of Pyridoxal Phosphates and Derivatives

A modified Varian HA-100 NMR spectrometer which enables studies of ^{31}P and ^{1}H chemical shifts by double resonance techniques is being used to study the role of phosphates of the Vitamin B_6 group in interaction with proteins. Preliminary results indicate a specific interaction that is being further investigated (R. Katz, H. Yeh, and D. F. Johnson).

Nuclear Magnetic Resonance Studies

A number of collaborative studies have been carried out (H. Yeh) in this laboratory using the Varian HA-100, which has been modified to do double resonance and variable temperature experiments. The following summarized studies are completed or in progress:

1. ^{1}H and ^{19}F Ring Fluorinated Imidazole Derivatives, and Intramolecular Interaction of (5-methyl-imidazole-4 yl) Dimethyl Butyric Acid and Analogs (K. Kirk, Y. Kikugawa, and L. Cohen).
2. Studies of Codeine and Isocodeine Derivatives (A. Jacobson and L. Sargent
3. Nucleophilic Additions to Arene Oxides, and Mono- and Di-Methyl Benzene Oxide-Oxepin Valence Tautomerism (A. Jeffrey, H. Yagi, and D. Jerina).
4. Stereochemical Dependence of NMR Parameters on Geometry in ^{15}N-E and Z Aldimines (H. Ziffer and D. Jerina).
5. Role of Metal Ions in Histidine Ammonialyase (C. Klee, K. Kirk, and L. Cohen).
6. Studies of Human Erythrocytes and Frog Muscle by ^{23}Na NMR. (E. Becker).

SECTION ON PHARMACODYNAMICS

The Role of Cyclic Adenosine Monophosphate in the Central Nervous System

The effects of various agents on levels of ATP, ADP, AMP and cyclic AMP in slices of guinea pig cerebral cortical grey matter, labeled by a prior incubation with radioactive adenine indicate that adenine is incorporated into morphological compartments containing adenine nucleotides that serve as the major source of nucleotide precursor for cyclic AMP. The nucleotides of this compartment represent less than one third of the total adenine nucleotides of the slice. The cyclic AMP that accumulates in labeled guinea pig cortical slices after stimulation with a variety of agents has approximately the same specific activity suggesting that all of the agents interact with similar compartments of labeled nucleotides. However, an initial transient increase in cyclic AMP levels elicited by histamine appears to take place in a compartment, whose adenine nucleotides are labeled to a much lower extent with radioactive adenine. Pulse labeling techniques with radioactive adenine reveal a high turnover rate for cyclic AMP which is proportional to the magnitude of the steady state level of the cyclic nucleotide. Adenosine stimulates accumulation of cyclic AMP and potentiates or reveals the stimulatory effect of biogenic amines. These effects on cyclic AMP accumulation are not potentiated by phosphodiesterase inhibitors that are quite effective in the absence of adenosine. Biogenic amines can elicit only one accumulation of cyclic AMP in brain slices, but combinations of adenosine and the biogenic amine are effective during a sequence of at least four restimulations. The specific activity of cyclic AMP in adenine labeled slices remains nearly constant during these sequential stimulations indicating that under conditions of repetitive conversions of at least 40% of the compartmentalized adenine nucleotides to cyclic AMP, significant translocation of nucleotides from less highly labeled compartments does not occur. An intermediate incubation of amine-stimulated slices with adenosine does not restore responsiveness to an amine. However, the presence of a phosphodiesterase inhibitor does partially restore amine-elicited accumulations of cyclic AMP during a second stimulation. (M. Huang and J. Schultz).

Responses of the cyclic AMP-generating system to biogenic amines are mediated *via* separate receptors for catecholamines, histamine and serotonin. In guinea pig cerebral cortical slices, a preponderance of α-adrenergic stimulatory receptors are present, while in rat both α- and β-receptors are present. In mouse primarily β-receptors are involved in catecholamine-mediated increases in cyclic AMP levels. Pretreatment of rats with 6-hydroxydopamine and a related substance, 2,3,5-trihydroxyphenethylamine, cause an increase in the maximal accumulation of cyclic AMP elicited by catecholamines, but not by other agents. Both α- and β-adrenergic receptor-mediated increases in cyclic AMP levels were potentiated by this pretreatment. Dihydroxytryptamines did not significantly alter the magnitude of cyclic AMP accumulations elicited in rat cortical slices by a variety of agents including serotonin. (M. Huang and J. Schultz).

Norepinephrine, serotonin and adenosine elicit accumulations of cyclic AMP in slices prepared from different neocortical areas and from limbic cortex of squirrel monkey. (M. Huang and P. Skolnick).

The accumulation of radioactive cyclic AMP in cortical slices, prelabeled with radioactive adenine is greater in the parent strain of mice from which the quaking mouse mutation was obtained. The lower accumulations of radioactive cyclic AMP in the quaking mouse appear to be due to alterations in the extent of incorporation of radioactive adenine into compartments whose nucleotides serve as precursors of cyclic AMP-generating system. (P. Skolnick).

Studies on Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

Purification of catechol-O-methyltransferase from the soluble fraction of rat liver has now afforded a single protein species with a molecular weight of 23700. Amino acid analysis indicates the presence of two cysteine moieties in this form of catechol-O-methyltransferase. Inactivation studies with ^{14}C-N-ethylmaleimide and other sulfhydryl reagents indicate that one sulfhydryl group is present in the active center of the enzyme and is related to the binding of S-adenosylmethionine. Another form of catechol-O-methyltransferase with an apparent molecular weight from 37000-46000 was also isolated. Antibodies have been prepared by administration of the low molecular weight form to rabbits. The antibodies cross-react with enzyme in crude liver microsomes prepared from rat, mouse and guinea pig and the same enzyme in a solubilized preparation from rat liver. (C. Creveling).

Long term destruction of the cardiac uptake mechanism and storage sites for norepinephrine, elicited by certain tri- and tetrahydroxyphenethylamines, is enhanced by monoamine oxidase inhibitors. In contrast, long term destruction elicited by 5,7- and 6,7-dihydroxytryptamines is prevented by inhibition of monoamine oxidase. 5,6-Dihydroxytryptamine does not cause destruction of the uptake mechanism and storage sites for norepinephrine, but both it and to a greater extent, 6,7-dihydroxytryptamine, cause necrotic lesions in both heart and blood vessels. The results indicate that destruction of cardiac noradrenergic terminals is dependent upon both a high affinity for neuronal uptake and a rapid rate of autoxidation in the phenethylamine series, while in the dihydroxytryptamine series a cytotoxic metabolite would appear to be formed by the action of monoamine oxidase. All of the dihydroxytryptamines autoxidize at a slow rate compared to that of 6-hydroxydopamine. Structural requirements for inhibition of cardiac uptake of radioactive norepinephrine have now been defined for both phenethylamines and tryptamines. 5,7-Dihydroxytryptamine has been found to be more active than 5,6-dihydroxytryptamine with respect to selective destruction of serotonin-containing neurons in the central nervous system. (J. Lundstrom and C. Creveling).

The Mechanism of Enzymatic Oxidation and the Role of Reactive Intermediates in Drug Metabolism, Hepatotoxicity and Carcinogenesis

Efforts to establish practical synthetic methods for the preparation of labile arene oxides have proved most rewarding. In addition to adaptive known synthetic methods for the elaboration of all the mono- and dimethylbenzene oxides as well as the oxide of 1,3,5-trimethylbenzene, an entirely new method has been developed which employs a latent epoxide precursor. The method is proving extremely adaptable to the synthesis of hithertofore inaccessible arene oxides -- particularly those of the polycyclic, carcinogenic hydrocarbons. (H. Yagi and D. Jerina).

Detailed examination of the mechanism by which arene oxides rearrange to phenols has shed considerable insight on their role in drug metabolism and cytotoxicity. Carbonium ions were shown to be intermediates in these reactions. The results allow firm predictions to be made as to biological lifetime of an arene oxide, ratio of product phenols which can be expected, and reactive intermediates such as new arene oxides and ketones which might be formed. Thus, a physical-chemical basis now exists for a priori prediction of the phenols, dihydrodiols, and glutathione conjugates that will form from a particular arene oxide. Interestingly, only the relatively stable arene oxides have been implicated in liver necrosis and carcinogenesis. (H. Yagi and D. Jerina).

In vivo studies of the metabolism of simple substituted benzenes have shown that compounds which have electron withdrawing groups show a primary isotope effect on hydroxylation at the position *meta* to the substituent. This observation is inconsistent with an arene oxide intermediate and suggests a direct insertion mechanism catalyzed by a different hepatic enzyme. (J. Tomaszewski).

Investigation of the hydroxylation of chlorobenzene with liver microsomes and with the soluble preparations of hepatic cytochrome P450 and P448, revealed a marked variation in the ratio of isomeric phenols. The *ortho*- and *para*-chlorophenols are formed respectively, from isomerization of the 2,3- and 3,4-arene oxides, while the *meta*-chlorophenol appears to be formed by a direct hydroxylation pathway (H. Selander).

Examination of the substrate specificity of glutathione-S-epoxide transferase have shown wide differences in reactivity of arene oxides -- those of polycyclic hydrocarbons being most reactive. These observations point to the importance of such enzymes as a protection mechanism against necrosis and carcinogenesis. (D. Jerina).

Solubilized epoxide hydrase has now been separated from catalytically active cytochrome P450, and preliminary observations and suggest that several hydrases are actually present based on differential purification factors.

Studies on the mechanism of action of the enzyme in liver microsomes indicate that steric rather than electronic factors are most important. (P. Dansette and D. Jerina).

Work in the area of physical organic chemistry has led to the first preparation of a *cis*-aldimine and has established both the sign and magnitude of H-C=^{15}N-coupling constants in these systems. This information is of value in structure determination and important to the general theory of spin-spin coupling. γ-Hydrogen rearrangement has been established as the principal mode of decomposition of the C_7H_8 + ion in the mass spectrometer by investigation of deuterium-labeled compounds. (D. Jerina).

Serial No. NIAMDD-LC-1
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Oxidation Mechanisms in Metabolic Processes

Previous Serial Number: SAME

Prinicipal Investigator: Louis A. Cohen

Other Investigators: Y. Kikugawa and Herman Yeh

Cooperating Units: Jack S. Cohen, DCRT-PSL-5.11

Man Years

Total: 1.0
Professional: 1.0
Other: 0

Project Description:

A study of the mechanisms by which energy derived from respiration is ultimately converted into labile bond energy, as in ATP; a study of the mechanisms by which molecular oxygen is activated for direct incorporation into substrate.

Objectives: To demonstrate that when oxygen is bound to metal-chelate systems, it is transformed from the triplet (diradical) state to the singlet (ionic) state and that the direct incorporation of oxygen into substrate involves the electrophilic attack of activated singlet oxygen. To study possible mechanisms for the conservation of oxidative energy by means of chemical models and to apply such knowledge to elucidation of the enzymatic pathways. To devise chemical models for the Mitchell hypothesis--that ATP formation can be coupled to a pH gradient and the free energy available therefrom.

Methods Employed: Infrared and ultraviolet spectroscopy are used to follow rates of reaction of intermediates and to determine structural characteristics. The latter method is also used for quantitative assay of labile materials. Nuclear magnetic resonance spectroscopy is used for structural elucidation of closely related materials. Various chromatographic procedures, particularly on thin layer plates, are used extensively.

Major Findings: Conformationally constrained imidazolecarboxylic acid esters hydrolyze by way of their cyclic acylimidazoles. Acylimidazoles have been implicated in a variety of other reactions of these systems, providing evidence that high energy bonds can be created enzymatically solely by the trapping of entropic free energy.

Significance to Bio-medical Research and the Program of the Institute: The mechanism by which molecular oxygen is carried into the cell, bound to hemoglobin, has been well elucidated. Its subsequent fate remains obscure. One immediate goal of this project is to determine the mechanisms by which oxygen is activated to a state in which it can attack a variety of relatively inert substrates.

Similarly, the function of ubiquinone in oxidative phosphorylation has yet to be elucidated. It has been shown that oxidative energy is utilized in the conversion of ADP to ATP in mitochondria, ubiquinone being an essential cofactor.

The present findings permit a novel role to be assigned to ubiquinone, Vitamin K and other quinone cofactors.

Controversy regarding the Mitchell hypothesis for mitochondrial synthesis of ATP has continued unabated, since no experiment has been devised to test its validity. The model system under study will be used to attempt to settle the question of whether ATP can be synthesized simply by a rapid change in pH.

Proposed Course: Studies will be continued on bond conservation of oxidative processes and efforts will be made to bridge the gap between the chemical model and the mitochondrion. Studies will be continued on the effects of considerable reduction in the electrostatic gap.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-2
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Chemical Modification and Cleavage of Proteins

Previous Serial Number: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: None

Cooperating Units: Robert Jerussi, FDA
Gerald Aurbach, NIAMDD-MD

Man Years

Total: 0.3
Professional: 0.3
Other: 0

Project Description:

A study of the chemical modification and fragmentation of polypeptides and proteins by use of selective chemical and physico-chemical reagents. The elucidation of sequence, tertiary structure and protein topology by such methods. Development of techniques for the selective radiolabeling of membrane proteins.

Objectives: To effect the selective modification and splitting of complex peptides by chemical means. To demonstrate the utility of the techniques in splitting long peptide chains into shorter fragments, facilitating sequence determination. To utilize such methods for selective modification of function groups on the surface of a globular protein and thus permit the probing of the surface structure. To modify proteins by selective oxidation or reduction techniques.

Methods Employed: Ultraviolet spectroscopy is used to follow the course and extent of cleavage reactions. Infrared spectroscopy is used to elucidate the structure of reaction products. Paper and column chromatography, ion exchange and high-voltage electrophoresis are used to separate and purify polypeptide fragments. An automatic amino acid analyzer is used to follow the extent of chemical reaction and the purification of peptide fragments. Gas chromatography assists in quantitative end-group assay. Various techniques are used for assay of enzyme activity, including spectral and automatic pH devices. Electrochemical oxidation is effected by use of controlled voltage equipment coupled with automatic pH control.

Major Findings: A technique has been developed for the conversion of carrier-free ^{125}I into the nonvolatile, stable, and powerful iodinating agent, N-iodosuccinimide. Production of this halogen carrier on a large scale will greatly facilitate the technique of radioimmune assay of biological materials.

A soluble polymer containing N-iodosuccinimide units has been developed, which is capable of selective iodination of highly exposed tyrosine and histidine in membrane protein. By use of radioactive iodine in this polymer, the identification of receptor protein may be facilitated.

Significance to Bio-medical Research and the Program of the Institute: Despite the remarkable achievements of the past several years in the X-ray crystallography of proteins, there exists no satisfactory method for determining three-dimensional structure in solution, under conditions of enzymatic activity. The use of a chemically reactive polymer or a platinum electrode, capable of selectively and rapidly modifying functional groups only on the surface, may provide a solution to the problem.

The ability to label membranes in a selective and specific manner with radioactive iodine will serve to facilitate studies on the sites and mechanisms of hormone binding to tissues.

Proposed Course: Information obtained by selective iodination, oxidation and reduction of proteins will be used to provide further information on conformation in solution.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-3
1. Chemistry
2. Biochemical
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cleavage of Peptide Bonds by Intramolecular Participation and Mechanisms of Action of Hydrolytic Enzymes

Previous Serial Number: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: None

Cooperating Units: None

Man Years

Total: 0
Professional: 0
Other: 0

This project has been temporarily discontinued.

Serial No. NIAMDD-LC-4
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Fluoro Analogs of Enzyme Substrates

Previous Serial Number: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: Kenneth L. Kirk, Wakatu Nagai, John Reepmeyer and Herman Yeh

Cooperating Units: Claude Klee, NIAMDD-LBP
Jack S. Cohen, CR-PSL
Dr. Michael Monahan, Salk Institute, LaJolla, California

Man Years

Total: 2.3
Professional: 2.3
Other: 0

Project Description:

Development of new methods for the synthesis of fluoro analogs of biochemically significant compounds and examination of their properties as enzyme inhibitors and as potential drugs.

Objectives: Fluoro analogs of steroids, purines, pyrimidines and amino acids have been synthesized and tested, several having been found valuable in arthritis and cancer chemotherapy. A larger number have not been prepared because of the limited routes available for the introduction of fluorine. Despite the ubiquitous role of imidazoles in biological systems (RNA, DNA, histidine, histamine, etc.), no fluorinated imidazole has yet been prepared. The initial purpose of this work was to develop a general method suitable for the preparation of fluoroimidazoles, to prepare a series of such compounds, and to test their abilities to serve as enzyme substrates or inhibitors and as replacement for histidine in proteins or for purines in DNA and RNA.

Methods Employed: Ultraviolet irradiation is used as the key step in the introduction of fluorine. Infrared, ultraviolet, mass, and nmr spectroscopy are used to follow the course of reactions and to elucidate product structure. Various chromatographic procedures are used for analysis and purification of materials.

Major Findings: The fluorine atom in 2-fluoroimidazoles has been found subject to displacement by strong nucleophiles. This phenomenon opens the door to testing of 2-fluoroimidazole analogs of substrates, hormones and nucleotides as affinity labels for enzyme active sites and membrane receptor sites. 2-Aminohistidine peptides have been shown to be substrates for tryptic cleavage, because the amino acid behaves geometrically and electronically like arginine in the active site. 2-Fluorohistamine has been found to simulate the action of histamine in stimulating the formation of cyclic AMP in brain tissue.

Significance to Bio-medical Research and the Program of the Institute: Past experience with fluoro analogs (fluorocitrate, fluorocortisone, and fluorouracil) warrants a search for other medicinally useful fluoroanalogs. Recent findings on the value of histidine in arthritis therapy, of imidazolecarboxamide in skin cancer therapy, and of histamine as a false neurotransmitter emphasize the magnitude of directions in which fluoroimidazoles may be examined clinically.

Proposed Course: Fluoroimidazoles already prepared will be submitted for pharmacological testing and will be examined as enzyme substrate analogs. Additional and more complex compounds will be prepared for the same purpose. The photochemical method of synthesis will be applied to other types of heterocyclic systems, in addition to imidazole. Fluorine nuclear magnetic resonance spectroscopy will be used to study enzyme-substrate complexes with a view to aiding in the elucidation of mechanism of enzymes such as histidine deaminase.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-5
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: General Principles of Enzyme Catalysis and Simulation

Previous Serial Number: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: Paul S. Hillery

Cooperating Units: None

Man Years

Total: 1.4
Professional: 1.4
Other: 0

Project Description:

A study of the principles used by enzymes to accelerate chemical reactions. Model compounds are designed and synthesized which are capable of undergoing various reactions at rates comparable to those of enzymes, and under the same reaction conditions. Recognition of the principal devices used by enzymes will permit the design of more effective drugs and enzyme inhibitors or substrates.

Objectives: The rates of enzyme-catalyzed reactions exceed those of simple test-tube analogs by factors of 10^{10}-10^{18}. In order to account for such a phenomenal effect, model compounds which duplicate one or more of an enzyme's special powers are studied to learn more about the vastly complex protein catalyst. In the belief that the principal enzymatic device is stereopopulation control (near perfect orientation of functional groups), model compounds have been designed and synthesized in which rotation of covalent bonds has been severely restricted. By bringing two functional groups into very close proximity and orientation, rates of reaction comparable to those of enzymes can be achieved in the test tube.

Methods Employed: Spectroscopic methods are used to elucidate the structures of synthetic products and to follow rates of reaction. Various chromatographic procedures are employed for analysis and purification.

Major Findings: In extension of our earlier studies, 2-benzofuranones have been synthesized in which the ground state is so similar to the transition state that no lactone ring opening can be observed. Pentamethylhomophthalic acid has been found to be converted into its anhydride with general acid catalysis. In this case, stereopopulation control creates a rate enhancement factor of 10^9 and an equilibrium enhancement factor of 10^6.

Honors and Awards: None

Publications:

Milstien, S. and Cohen, L. A.: Stereopopulation Control. I. Rate enhancement in the lactonization of o-hydroxycinnamic acids. J. Amer. Chem. Soc., 94: 9158-9165, 1972.

Borchardt, R. T. and Cohen, L. A.: Stereopopulation Control. II. Rate enhancement of intramolecular nucleophilic displacement. J. Amer. Chem. Soc., 94: 9166-9174, 1972.

Borchardt, R. T. and Cohen, L. A.: Stereopopulation Control. III. Facilitation of intramolecular conjugate addition of the carboxyl group. J. Amer. Chem. Soc., 94: 9175-9182, 1972.

Kirk, K. L. and Cohen, L. A.: Intramolecular aminolysis of amides. Effects of electronic variation in the attacking and leaving groups. J. Amer. Chem. Soc., 94: 8142-8147, 1972.

Takahashi, S., Cohen, L. A., Miller, H. K., and Peake, E. G.: Calculation of the pK_a values of alcohols from σ^* constants and from the carbonyl frequencies of their esters. J. Org. Chem., 36: 1205-1209, 1971.

Cohen, L. A. and Takahashi, S.: A reexamination of electronic effects in ring-substituted phenyl esters. Correlation of spectral and kinetic properties with σ^0. J. Amer. Chem. Soc., 95: 443, 1973.

Serial No. NIAMDD-LC-6
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the Synthesis of Carbohydrate Derivatives for Biomedical Research

Previous Serial Number: NIAMD-LC-8

Principal Investigator: H. G. Fletcher, Jr.

Other Investigators: H. W. Diehl, A. Hasegawa (to 9/30/72), M. Pokorny (from 11/28/72), R. D. Ness, E. W. Tracy, E. Zissis

Cooperating Units: Dr. N. Pravdić, Department of Organic Chemistry and Biochemistry, "Rudjer Bošković" Institute, Zagreb, Yugoslavia (under P.L. 480)

Man Years

Total: 7.3
Professional: 4.3
Other: 3

Part A. 2-Acetamido-2-deoxyaldonic Acids

Project Description:

Objective: To investigate the chemistry and biochemistry of the 2-acetamido-2-deoxyaldonic acids and their derivatives.

Major Findings: Three chemical processes have been developed which have permitted the isolation of the first-known 2-acetamido-2-deoxyhexonic acids—a new class of substance representing the aldonic acids related to the biochemically important *N*-acetylaminosugars. These structures are comparatively stable to alkali and thus 2-acetamido-2-deoxy-D-mannonic acid can be converted into its hitherto unknown dicyclohexylammonium salt through the action of aqueous dicyclohexylamine. In contrast to these free acids, the corresponding 2-acetamido-2-deoxyaldonolactones are markedly labile in the presence of bases. Thus 2-acetamido-2-deoxy-D-mannono-1,4-lactone affords a mixture of the dicyclohexylammonium salts of 2-acetamido-2-deoxy-D-mannonic acid and 2-acetamido-2-deoxy-D-gluconic acid when treated with aqueous dicyclohexylamine. With nonaqueous dicyclohexylamine, the lactone undergoes a β elimination to give 2-acetamido-2,3-dideoxy-D-*erythro*-hex-2-enono-1,4-lactone. It is now clearly evident that the 2-acetamido-2-deoxyaldonic acids and their derivatives have many interesting properties which are not shown by the much better known aldonic acids having a hydroxyl group at carbon 2.

Significance to Biomedical Research and the Program of the Institute: Our understanding of the chemical properties of the 2-acetamido-2-deoxyaldonic acids and their derivatives has been expanded as a preliminary step toward studies of the biochemical properties of these substances.

Part B. Unsaturated Aminosugars

Project Description:

Objective: To study the chemical properties of a new and unique class of carbohydrate derivative, the *N*-acyl- and di-*N*-acylenamines which are related to the common *N*-acetylhexosamines.

Major Findings: Earlier studies have shown that various nucleophiles react with 3,4,6-tri-*O*-acetyl-2-(*N*-acetylacetamido)-1,5-anhydro-2-deoxy-D-hex-1-enitols when heated in the presence of an acid catalyst to give 4,6-di-*O*-acetyl-2-(*N*-acetylacetamido)-2,3-dideoxy-α-D-hex-2-enopyranose derivatives. The overall transformation appeared to arise from a nucleophilic attack on carbon 1, accompanied by loss of the acetoxy group at C-3 and allylic shift of the double bond. Subsequent studies have now shown that this view of the mechanism represents an oversimplification. It now appears that the starting product undergoes a preliminary series of rearrangements, the acetoxy group at C-3 migrating with allylic shift to the β side of C-1, followed by anomerization to the thermodynamically more stable 1,4,6-tri-*O*-acetyl-2-(*N*-acetylacetamido)-2,3-dideoxy-α-D-hex-2-enopyranose. The C-1 acetoxy group in this product is then displaced by the nucleophile to yield the observed final product.

Two pairs of anomers, the 1,4,6-tri-*O*-acetyl-2-(*N*-acetylacetamido)-2,3-dideoxy-D-hex-2-enopyranoses of the *erythro* and *threo* series have been prepared and characterized. Nuclear magnetic resonance studies of these structures suggest that the α-D-anomers exist in the $\underline{H}_5^{\circ}$ conformation while the β-D anomers assume a conformation which is closer to $\underline{H}_5^{\circ}$ than $\underline{H}^5_{\circ}$.

Studies of the behavior of a variety of 1,4,6-tri-*O*-acyl-2-(*N*-acetylacetamido)-[and acetamido]-2,3-dideoxyaldohex-2-enoses with hydrogen in the presence of a palladium catalyst have revealed a remarkable specificity. While β-D anomers are not attacked, all α-D anomers (with one exception) undergo allylic hydrogenolysis at C-1. An interpretation of this unusual situation has been proposed.

A simple method for the conversion of *N*-acetylacetamido derivatives to acetamido compounds (*i.e.* a mono-N-deacetylation) has been devised.

Significance to Biomedical Research and to the Program of the Institute: This project continues to provide the sort of background information which is essential to the ultimate synthetic object, namely the preparation of certain new types of potentially cytotoxic substances.

Part C. The Isopropylidenation of Certain Aldohexoses

Project Description:

Objective: To develop a new type of substitution in aldohexoses and 2-acetamido-2-deoxyaldohexoses.

Major Findings: When treated at room temperature in *N*,*N*-dimethylformamide solution and in the presence of a trace of *p*-toluenesulfonic acid with a small molar excess of 2,2-dimethoxypropane, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose, 2-acetamido-2-deoxy-D-galactose and D-glucose gave the corresponding 4,6-*O*-isopropylidenealdopyranoses. All of these derivatives are new and are not given by any of the older isopropylidenating reagents. D-Mannose, on the other hand, affords the known 2,3:5,6-di-*O*-isopropylidene-D-mannofuranose. The yields from these reactions are high and the structures of the new products have been elucidated unequivocally.

When the reagent mixture is used at 80° the results are almost totally different. 2-Acetamido-2-deoxy-D-mannose gives a mixture from which methyl 2-acetamido-2-deoxy-2,3-*N*,*O*-isopropylidene-5,6-*O*-isopropylidene-α-D-mannofuranoside, 2-acetamido-2-deoxy-2,3-*N*,*O*-isopropylidene-5,6-*O*-isopropylidene-D-mannofuranose, and methyl 2-acetamido-2-deoxy-5,6-*O*-isopropylidene-α-D-mannofuranoside were isolated. 2-Acetamido-2-deoxy-D-galactose gave compounds which were identified as methyl 2-acetamido-2-deoxy-5,6-*O*-isopropylidene-β-D-galactofuranoside and methyl 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-β-D-galactopyranoside. 2-Acetamido-2-deoxy-D-glucose yielded methyl 2-acetamido-2-deoxy-5,6-*O*-isopropylidene-β-D-glucofuranoside and methyl 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-β-D-glucopyranoside. All of these products represent hitherto unknown structures. Under these more vigorous conditions D-mannose gave the known 2,3:5,6-di-*O*-isopropylidene-D-mannofuranose although in notably higher yield than had been found with the milder conditions.

Significance to Biomedical Research and to the Program of the Institute: These wholly new carbohydrate derivatives offer great potential for the synthesis of hitherto inaccessible structures.

Honors and Awards: None

Publications:

Glaudemans, C. P. J. and Fletcher, H. G., Jr.: 2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranose. In Whistler, R. L. and BeMiller, J. N. (Eds.): Methods in Carbohydrate Chemistry. New York, Academic Press, 1972, vol. 6, pp. 373-376.

Zissis, E., Diehl, H. W., and Fletcher, H. G., Jr.: Dicyclohexylammonium Salts for the Isolation and Characterization of Aldonic Acids. Carbohyd. Res. in press.

Pravdic, N., Zidovec, B. and Fletcher, H. G., Jr.: On the Mechanism of the Rearrangement of Some Acetylated Unsaturated 2-Acetamidoaldose Derivatives. Selective Removal of one *N*-acetyl Group from 2-(*N*-Acetylacetamido) Compounds. *Croat*. *Chem*. *Acta* in press.

Pravdic, N., Zidovec, B., Franjic, I., and Fletcher, H. G., Jr.: Catalytic Hydrogenation of Some 2-Acetamidoaldose Derivatives. *Croat*. *Chem*. *Acta* in press.

Zissis, E., Diehl, H. W., and Fletcher, H. G., Jr.: The Preparation and Properties of Some 2-Acetamido-2-deoxyhexonic Acids. *Carbohyd*. *Res*. in press.

Hasegawa, A., and Fletcher, H. G., Jr.: The Behavior of Some Aldoses with 2,2-Dimethoxypropane—*N*,*N*-dimethylformamide—*p*-toluenesulfonic Acid. I. At Room Temperature (~25°). *Carbohyd*. *Res*. in press

Hasegawa, A., and Fletcher, H. G., Jr.: The Behavior of Some Aldoses with 2,2-Dimethoxypropane—*N*,*N*-dimethylformamide—*p*-toluenesulfonic Acid. II. At 80°. *Carbohyd*. *Res*. in press

Serial No. NIAMDD-LC-7
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunochemistry and Reactions of Carbohydrates

Previous Serial Number: NIAMD-LC-7

Principal Investigator: C. P. J. Glaudemans

Other Investigators: M. E. Jolley

Cooperating Units: NCI-5556

Man Years

Total: 1.8
Professional: 1.8
Other: None

Subtitle: Carbohydrate Antigen-Binding Meyloma Immunoglobulins in Mice (with Dr. M. Potter)

Project Description:

Objectives: This laboratory is interested in the interaction of carbohydrate antigens with antibodies. Last year we reported finding two homogeneous myeloma immunoglobulins from mice (proteins S-10 and T191B) which interact specifically with a polysaccharide antigen (aβ-D-galactopyranose polymer).

We now wish to report three more IgA myeloma immunoglobulins with the same antigalactan specificity (proteins X-24, X-44 and J-1). A sixth IgA protein (J-539), previously reported by others, was shown to bind our antigen specifically, also. Thus we have here six homogeneous immunoglobulins, all different (see later), all having their active sites directed towards multiple units of β-D-galactopyranose. This group of IgA's provides a unique set of homogeneous immunoglobulins, structurally diverse, but all directed towards an extremely simple carbohydrate determinant involving sequences of only one neutral monosaccharide moiety and only one type of linkage. The study of this system should greatly clarify our understanding of immunochemical interactions.

Methods employed: The usual techniques of chemistry and biochemistry are employed such as affinity chromatography employing hapten-coupled Sepharose columns, fluorimetry, organic synthesis, gas liquid chromatography, mass spectroscopy, and chromatography in general.

Major Findings:

A. Fluorescence titrations: Proteins are initially detected in routine screening versus an arabinogalactan. All six IgA's were isolated from ascites by affinity chromatography employing Sepharose-bovine serum albumin-galactoside columns. These immunoglobulins were shown to be single substances (immunoelectrophoresis). Their interaction was studied with a series of oligosaccharide ligands which form the structural repeating unit of the carbohydrate antigen, namely β1→6 galactodiose (Gal_2), triose (Gal_3), and tetraose (Gal_4). These oligosaccharides were isolated from the partial hydrolysis of gum ghatti followed by extensive chromatography. The binding of the corresponding Fab fragments (reduction and alkylation followed by pepsin digestion and Sephadex chromatography) with the above ligands was also studied.

Earlier work had shown that the precipitation of proteins S-10 and T-191 by arabinogalactan on agar double diffusion was increasingly inhibited in the order: methyl β-D-galactoside $<Gal_2 <Gal_3 <Gal_4$. The greatest change in inhibition resulted in going from methyl β-D-galactopyranoside to Gal_2, and the difference between Gal_3 and Gal_4 was small.

Some antibody-antigen interactions are known to involve changes in the native fluorescence of the antibody. Interactions of proteins with a ligand usually, but not always, involve fluorescence quenching. Fluorometric titrations of our immunoglobulins following the addition of ligands (Gal_2, Gal_3, and Gal_4) revealed that two proteins (X-24 and J-539) demonstrated increased intensity of fluorescence with Gal_3 and Gal_4, but not with Gal_2. No immunoglobulin has hitherto been found to show an increase in fluorescence intensity upon binding with ligand.

In addition, one immunoglobulin (X-44) showed quenching upon the addition of ligands Gal_3 and Gal_4. Again no changes were observed upon the addition of Gal_2. We assume that the relative increase in fluorescence intensity is directly proportional to the fraction of available sites occupied by ligand. This observation can then be used to measure accurately the constant of association between ligand and protein. This assumption is only valid if a) all sites have the same affinity for ligand and b) the binding of ligand at one site does not affect the binding of ligand at another site. These assumptions are borne out by the data.

Since the ligand is non absorbing in the UV spectrum and carries neither a charge nor an aromatic grouping, fluorescence changes must have their origin in the protein molecule. Almost certainly these must involve a change in the microenvironment of one or more tryptophan residues.

Hg (II) ions react specifically with tryptophan residues in proteins at high pH and produce particular spectral changes. At pH 4.5 no change is observed. Sulphydryl groups are also reactive but show no such pH dependence and no such spectral changes. The observation of such a spectrum upon adding Hg (II) ions to proteins X-24, X-44 and J-539 indicates the presence of an accessible tryptophan residue(s) in the molecule. The absence of such a spectrum in these proteins when Hg (II) is added in the presence of ligand

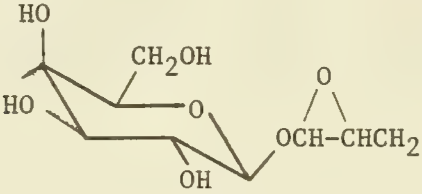
(Gal$_3$ and Gal$_4$) strongly suggests that this accessible tryptophan residue is actually located in the active site. Furthermore, those three immunglobulins showing this fluorescence change also exhibit turbidity upon the addition of Hg (II) ions, a phenomenon that is completely inhibited by the prior addition of low molar amounts of ligands Gal$_3$ and Gal$_4$ (but not Gal$_2$). Moreover, even after turbidity has occurred the addition of Gal$_3$ or Gal$_4$ can substantially clarify the suspension in the cases of proteins X-24 and J-539 globulins, and completely clarify the suspension of protein X-44. I addition, no precipitation was observed on exposing proteins X-24, J-539 and X-44 to Hg (II) at pH 4.5, indicating that the precipitation was not due to reaction with sulfhydryl groups in the protein, but with one or more tryptophan residues alone. Finally, the observed change in fluorescence intensity upon binding Gal$_3$ and Gal$_4$ ligand is enhanced for the Fab' fragment obtained from proteins X-24 and J-539 eliminating the F$_c$ portion as a locus for the tryptophan residue in question. From the above, it must be concluded that 1) the tryptophan residue(s) responsible for changes in fluorescence is(are) most likely located in the active site and 2) if this is so, this tryptophan residue is then probably located near the third subsite for the ligand from the terminal nonreducing galactosyl residue.

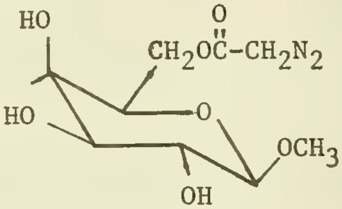
The magnitude of the association constants found by us for Gal$_3$ and Gal$_4$ with X-24 and J-539 and their Fab' fragments is between 1.5-3.5 x 10^5. This is very similar to the K$_a$ found by Spyer and Pappenheimer for an anti-pneumoccal polysaccharide (IgG of restricted heterogeneity) isolated from rabbits following immunization with pneumoccoi (2.5 x 10^5). The K$_a$ is much higher than that found by Sher and Tarikas for protein J-539 with isopropyl 1-thio-β-D-galactopyranoside (2.2 x 10^3, a result which is consistent with our findings.

There seems to be little difference in binding characteristics between Gal$_3$ or Gal$_4$ with X-24 and J-539. This general levelling off after the ligand size has reached the trisaccharide stage has also been observed by others in the case of an anti-α (1→3) dextran IgM immunoglobulin. This result appears unlike that of Kabat, who observed in the case of nonhomogeneous human anti-dextran, that maximum binding occurred at the hexasaccharide level, as indicated by inhibition studies. It is interesting to speculate on the reason for this difference. Inspection of a model of Gal$_4$ and a model of α(1→6) linked glucotetraose, shows that the angle between adjacent α-(1→6) glucosyl units needed to keep the intersaccharidic oxygen-atom and ring oxygen atom from orbital overlap makes isomaltotetraose more compact than a β-(1→6) linked hexosyl oligosaccharide, such as Gal$_4$. Although this is very speculative, it may be that combining sites accommodating Gal$_4$ are of a similar size to one accommodating the isomaltohexaose ligand whose size was proposed in the pioneering work by Kabat.

B. Affinity labeling: p-Aminophenyl-β-D-thiogalactopyranoside is an effective inhibitor of the interaction of the antigalactans with the antigen. The derived N-bromoacetate may interact with these proteins, but causes precipitation. Our search for appropriate affinity labeling compounds was then extended to two other possibilities. 1,2-Epoxypropyl-β-D-galactopyranoside was prepared from allyl-β-D-galactoside. It was found to be an effective inhibitor. Future work includes the preparation of this compound bearing a radioactive label.

The 6-O-glycyl derivative of methyl-β-D-galactopyranoside was prepared. Following that diazotization will yield a compound which can be transformed into a carbene in the presence of the antibody (e.g. in the active site) and can hopefully be inserted in the amino acid chain. The advantage of these two derivatives are that they are reactive affinity-labeling compounds without bulky groups, and are therefore closely related to the monosaccharide moiety as it actually occurs in the antigen.

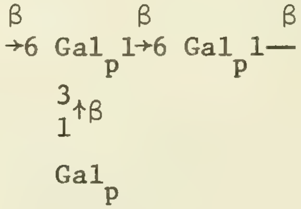




C. Partial Amino acid Sequence and Idiotype (with Dr. Potter's laboratory): Partial amino acid sequences of the V_{kappa} polypeptide of these six antigalactan IgA's to residue Cys23 and the V_{Heavy} polypeptide to residue 30 were determined on the automated sequences. No differences were found among the six V_k sequences. Among some 50 partial V_k sequences that have thus far been determined these six chains are the only ones thus far identified in this group. The V_H partial sequences were also very closely related but two differences were found. One protein differed from the other five by having isoleucine at position 5, a second protein differed from the others by having an unidentified amino acid at position 19. Using the highly sensitive inhibition of hemagglutination method it was found that each of the proteins possessed a unique individual antigenic specificity (all six have their own idio type).

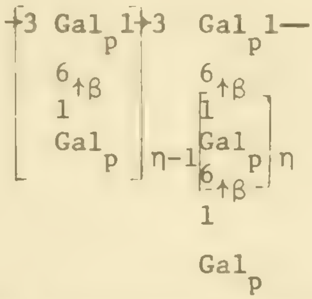
D. X-Ray Crystallographic Work: IgA J-539 has crystallized well enough for X-ray crystallographic work (Dr. Davies' laboratory). We have prepared tritium labeled ligand (Gal_3 and Gal_4) to study binding constants of the crystalline material so as to ensure structural kinship between crystals and solution-IgA.

E. Structure of Beef Lung Galactan: Beef lung galactan precipitates all IgA's with β1→6 galactan specificity. The structure proposed for BLG:



$$\begin{array}{l} \overset{\beta}{\rightarrow} 6\ \mathrm{Gal}_p\ 1\overset{\beta}{\rightarrow} 6\ \mathrm{Gal}_p\ 1\overset{\beta}{-} \\ \quad {}^{3}_{1}\uparrow\beta \\ \quad \mathrm{Gal}_p \end{array}$$

All our IgA's are precipitated by arabinogalactan which has short branches of multiple β1→6 galactose units. Since terminal units are undoubtedly imminodominant it appears that BLG probably has a 1,3 backbone instead, with short β1→6 branching:



This structure is equally well supported by the methylation data now in the literature. The structure of these polysaccharides becomes of interest when it is realized that a number of saline extracts from human lung tumors precipitate with these antigalactan IgA's.

Significance to Biomedical Research and the Program of the Institute: The Section on Carbohydrates, with its know-how of immunochemistry and structural carbohydrate chemistry is in a position to make a real contribution to knowledge of the genetics and the diversity of the immune response, employing this unique set of immuno globulins. The specificity of antibodies and the problem of "the economy of the immune response" can be studied very well using this set of immunoglobulins.

Proposed Course of Project: Binding constants for all IgA's will be studied with ligands. Synthetic ligands will then be modified stepwise to see how specific antibodies really are (economy of the immune response) affinity labeling with radioactive ligands followed by amino acid sequencing of the labeled IgA, together with X-ray crystallographic investigation, will lead to a mapping of the active site.

Honors and Awards: None

Publications:

Potter, M. and Glaudemans, C. P. J.: Homogeneous Mouse Immunoglobulins that Bind Carbohydrates. In Colowick, S. P. and Kaplan, N. O. (Eds.) Methods in Enzymology, New York, Academic Press, 1972, p. 388.

Jolley, M. E., Rudikoff, S., Potter, M., and Glaudemans, C. P. J.: Spectral Changes on Binding Oligosaccharides to Murine IgA Myeloma Protein. Biochemistry, in press.

Mushinski, E., Rudikoff, S., Potter, M. and Glaudemans, C. P. J.: Six Balb/c IgA Myeloma Proteins that Bind β(1→6)-D-galactans. Partial Amino Acid Sequences and Idiotypes. J. Immunol. in prepn.

Glaudemans, C. P. J., Jolley, M. E. and Potter, M.: The Structure of Mammalian Lung Galactan. J. Immunol. in prepn.

Serial No. NIAMDD-LC-8
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Testing for Analgesic Activity and Dependence Liability

Previous Serial Number: NIAMDD, LC, #16

Principal Investigator: Everette L. May

Other Investigators: E. L. Atwell, E. McNeal, and N. B. Eddy (consultant)

Cooperating Units: Animal Production Section, NIH; Department of Pharmacology, University of Michigan

Man Years:
Total: 1.5
Professional: 0.5
Other: 1.0

Project Description:

Objectives: 1) To determine analgesic activity, acute toxicity, narcotic antagonist activity and abuse potential of new compounds prepared in the Section on Medicinal Chemistry and in laboratories throughout the world. 2) To serve in an advisory capacity to the BNDD, FDA, WHO, SAODAP and the pharmaceutical industry.

Methods Employed: Hot-plate and Nilsen methods of assay for analgesia and 24-hour acute toxicity determinations using in all instances male, white, Caeserian-Derived, General-Purpose mice. Abuse potential and properties of narcotic antagonism were determined in Rhesus monkeys and, if warranted, in man at the Addiction Research Center, Lexington, Kentucky.

Major Findings: Eighty-six new compounds, about 45 from the (world-wide) pharmaceutical industry, the remainder from universities and our own laboratory were tested for analgesic acticity by the hot-plate method, sc (and occasionally oral), administration. Of these, 33 were considered of sufficient interest for complete assays. Forty-seven of the above compounds were also tested by the Nilsen procedure, generally more diagnostic for the agonist-antagonist type analgesic. Efforts to develop a simple, predictive method (using the Nilsen method for determining analgesia) for narcotic antagonistic effect have failed thus far.

Several potent, long-acting agonists (which would not support morphine dependence in monkeys) and orally effective, long-acting antagonists have been uncovered in these studies and in research conducted subsequently at the University of Michigan.

Significance to Biomedical Research and the Program of the Institute: Constitutes a concerted effort in attempts at the solution of the problem of drug addiction.

Proposed Course of Project: To continue essentially as is with possible addition of simple primary tests for physical dependence capacity, antagonistic action and antiinflammatory activity (all in the mouse).

Publications: None.

Serial No. NIAMDD-LC-9
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: 1) An alternative synthesis for 3,6-dimethyl-8-hydroxy-1,2,3,4,5,6-hexahydro-3-benzazocine and the synthesis of some new 3-alkyl analogs.
2) An alternative synthesis for 5-(m-hydroxyphenyl)-2-methylmorphan and the synthesis of some new 2-alkyl analogs.

Previous Serial No: Same

Principal Investigator: Michael E. Rogers

Other Investigators: Everette L. May

Cooperating Units: Microanalytical Services & Instrumentation Section

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: 1) To develop a more practical synthesis for 3,6-dimethyl-8-hydroxy-1,2,3,4,5,6-hexahydro-3-benzazocine and to prepare the 3-*n*-propyl through the 3-*n*-heptyl analogs to be evaluated as narcotic agonists-antagonists.

2) To develop a more practical synthesis for 5-(*m*-hydroxyphenyl)-2-methylmorphan and to prepare the 2-*n*-propyl through the 2-*n*-heptyl analogs to be evaluated as narcotic agonists-antagonists.

Methods Employed: Synthetic techniques, tlc, glc, mass, nmr, and ir spectroscopy.

Major Findings: 1) A novel synthesis of the benzazocines and one reported in a patent were investigated. The key intermediate for the novel synthesis, N-cyanomethyl-N-methyl-3-(*m*-methoxyphenyl)-butylamine was prepared in three steps with an overall yield of 25%. However, cyclization attempts with this and prior intermediates using polyphosphoric acid were unsuccessful. The key intermediate for the reported sysnthesis, N-(*p*-methoxyphenethyl)-N-tosyl-3-aminopropionic acid, was prepared in three steps in an overall yield of 83%. However, attempted cyclizations using this and other intermediates with polyphosphoric acid or aluminum chloride were unsuccessful. Communication with the authors of the patent revealed they were also unable to cyclize this specific intermediate.

2) The 3-*n*-propyl and 3-*n*-hexyl analogs of 3,6-dimethyl-8-hydroxy-1,2,3,4,5,6-hexahydro-3-benzazocine were preapred by known procedures from an available supply of 8-methoxy-6-methyl-1,2,3,4,5,6-hexahydro-3-benzazocine hydrochloride.

3) A novel synthesis of 5-(*m*-methoxyphenyl)-2-methylmorphan was developed. 2-(2-Hydroxyethyl)-2-(*m*-methoxyphenyl)-cyclohexanol was prepared by LAH reduction of 2-carbethoxymethyl-2-(*m*-methoxyphenyl)-cyclohexanone. 2-Formylmethyl-2-(*m*-methoxyphenyl)-cyclohexanone was prepared by Sarett oxidation of the diol. Treatment of the dicarbonyl compound with NaOH in THF gave 6-hydroxy-1-(*m*-methoxyphenyl)-8-oxobicyclo[3.2.1]octane which underwent Wolff-Kishner reduction to give 6-hydroxy-1-(*m*-methoxyphenyl)-bicyclo[3.2.1]octane. The alcohol was oxidized with Sarett's reagent to 1-(*m*-methoxyphenyl)-6-oxobicyclo[3.2.1]octane and the oxime made which underwent a Beckmann rearrangement to afford 5-(*m*-methoxyphenyl)-3-oxo-2-azabicyclo[3.3.1]nonane. Methylation of the lactam with methyl iodide and sodium amide gave 5-(*m*-methoxyphenyl)-2-methyl-3-oxoazabicyclo[3.3.1] nonane. Reduction with diborane gave 5-(*m*-methoxyphenyl)-2-methylmorphan. Overall yield for the scheme is 0.6%.

Significance to Biomedical Research and the Program of the Institute:

1) More efficient syntheses of the benzazocines and the morphans would make these compounds more attractive as useful analgetics.

2) The incorporation of alkyl chains greater than ethyl in other ring systems has conferred some antagonist activity to the compounds. It is hoped that this will be the case with the benzazocines and the morphans.

Proposed Course: To be continued as described.

Publications: None

Serial No. NIAMDD-LC-10
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Narcotic Antagonists.

Previous Serial Number: None

Principal Investigator: Everette L. May

Other Investigators: None

Cooperating Units: Department of Pharmacology, University of Michigan

Man Years:
Total: 0.5
Professional: 0.5
Other: 0

Project Description:

Objectives: To develop improved narcotic antagonists as deterrents to heroin abuse.

Major Findings: The levo antipode of α-(±)-2-allyl-5,9-dimethyl-2´-hydroxy-6,7-benzomorphan (I), an antagonist similar to nalorphine proved to be three times as potent as nalorphine with a similar duration of action. The (+)-isomer as expected was devoid of properties of antagonism but showed non-narcotic CNS depressant effects with marked ataxia not reversed by nalorphine. The (-)-isomer was not considered a worthwhile candidate for study as a deterrent to narcotic abuse. These antipodes were prepared from corresponding α-5,9-dimethyl-2-hydroxy-6,7-benzomorphans (alkylation with allyl bromide). Attempts to resolve I were unsuccessful.

Significance to Biomedical Research and the Program of the Institute: Consistent with efforts fostered by the SAODAP group to find a long-acting, orally effective narcotic antagonist.

Proposed Course: Project finished.

Publications: Ong, H. H. and May, E. L.: Photocyclizations. III. Synthesis of 3,6-dimethyl-8-hydroxy-3,4,5,6-tetrahydro-3-benzazocin-2(1H)-one. J. Org. Chem. 38: 924-927 (1973).

May, E. L.: The Methadone Story. Aldrichimica acta. 5: 38-40, 1972.

May, E. L.: Research toward non-abusive analgetics in Psychopharmacological Agents. Volume III. (M. Gordon, Ed), Chapter XII, Academic Press, New York, N. Y. (in press)

Eddy, N. B. and May, E. L : The search for a better analgesic. Implications for drug dependence of the morphine type. Science (in press).

Eddy, N. B. and May, E. L.: Origin and history of antagonists. First International Conference on Narcotic Antagonists, Warrenton, Virginia. Raven Press, New York, N. Y. (in press).

Jacobson, A. E. and May, E. L.: Perspectives in the Chemistry of Narcotic Antagonist. First International Conference on Narcotic Antagonists, Warrenton, Virginia. Raven Press, New York, N. Y. (in press).

Serial No. NIAMDD-LC-11
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: 1) 9-Hydroxybenzomorphan narcotic antagonists and analgesics.
2) Novel synthetic approaches to 5,9-dimethyl-9-hydroxy-2´-methoxybenzomorphan.
3) Mechanism of action of Δ^1-tetrahydrocannabinol from marihuana.

Previous Serial Number: None

Principal Investigator: Raymond S. Wilson

Other Investigators: Everette L. May

Cooperating Unit: Microanalytical Services & Instrumentation Section

Man Years:
Total: 1
Professional: 1
Others: 0

Project Description:

Objectives: 1) Preparation of various N-alkyl derivatives in the 2´,9-dihydroxy-benzomorphan series and subsequent testing as narcotic antagonists and analgetics.

2) Examination of novel synthetic routes to 9-hydroxybenzomorphans with particular emphasis on methods which would avoid N-demethylation steps prior to final N-alkylation.

3) Preparation and biological testing of 7-nor-Δ^1-THC and 7-nor-Δ^6-THC in an attempt to see if hydroxylation at the 7-methyl is actually necessary for activity as has been suggested in the literature.

Methods Employed: Standard organic chemical procedures including mass spectrographic and NMR techniques.

Major Findings: 1) The known method in the literature for preparation of 5-methyl-α-9-hydroxy-2´-methoxybenzomorphan, the immediate precursor of the desired products, was followed and various amounts of the intermediates accumulated. The erratic and frequently low yield dequaternization and N-demethylation steps prompted exploration of alternate synthetic methods.

2) 7-Methoxy-β-tetralone was converted into 2,3-(3´-methoxybenzo)-1,8-dimethyl-8-hydroxybicyclo-[3.2.1]-oct-2-en-6-one in several steps and the latter could be converted into the corresponding 3-oxo-benzomorphan via the Schmidt reaction. The yields of the desired product were generally less than 40% and the main product was 1,2-dimethyl-7,8-(3´-methoxybenzo)-bicyclo-[3.3.0]-octa-2,7-dien-4-one from acid catalyzed rearrangement. Prior acetylation of the alcohol gave essentially the same results under a variety of experimental conditions.

3) dl-6aβ,7,10,10aα-Tetrahydro-1-hydroxy-6,6-dimethyl-3-pentyl-6H-dibenzo[b,d]pyran-9(8H)-one was prepared using modifications of a reported procedure. This compound could be reduced to the corresponding alcohol in high yield with either $NaBH_4$ or $LiAlH_4$. Acid catalyzed dehydration produced what appears to be the desired 7-nor-Δ^6-THC based on its nmr and it would be the expected product by analogy to the 7-methyl series. Further examination of the nmr spectrum with Europium shift reagents and comparison with the 7-nor-Δ^1-THC should help confirm this assignment. Conversion of the alcohol to the corresponding chloro compound was accomplished by two different methods, however, both yields were about 40%. Formation of the phenolate anion with a hindered base followed by intramolecular dehydrohalogenation should give 7-nor-Δ^1-THC by analogy to the 7-methyl series. Attempted formation of the chloro compound from the alcohol using the Lucas reagent gave selective acetylation of the alcohol over the phenol. Attempted tetrahydropyranylation of the previously mentioned phenolic ketone prior to reduction and halogen formation resulted in C-alkylation ortho to the phenol, possibly a novel reaction of vinyl ethers.

Significance to Biomedical Research and the Program of the Institute:
2) A novel synthetic route to the benzomorphan nucleus was devised which would probably be of greater use in less acid labile systems. One of the intermediates in this route, 1-methyl-2,3-(2´-methoxybenzo)-6-hydroxy-bicyclo-[3.3.1]-oct-2-en-8-one, appears to be a useful intermediate for conversion to 6-methyl-1,2,3,4,5,6-hexahydro-8-methoxy-3-benzazocine in three synthetic steps.

3) Biological testing of 7-nor-Δ^6-THC and 7-nor-Δ^1-THC should help to settle the controversy in the literature over whether Δ^1-THC and Δ^6-THC are themselves active or must first be metabolized to the 7-hydroxy compounds before they are active. Also, the several structural analogs of Δ^1-THC prepared thus far will be tested for possible use as non-addicting sedative-hypnotics, tranquilizers, and analgesics.

Proposed Course: 1) Complete synthesis and testing of the desired compounds, and try alternate methods of N-demethylation such as chloroformates.

2) Attempt conversion of the previously mentioned intermediate to the benzazocine structure.

3) Continue synthetic approach to 7-nor-Δ^1-THC. Also, prepare 7-2mino-Δ^1-THC, the nitrogen analog of 7-hydroxy-Δ^1-THC, and examine it for analgesic and other biological properties.

Publications: None

Serial No: NIAMDD-LC-12
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Titles: 1) Molecular Orbital Studies of biologically important molecules.
2) Hansch analysis of catechol O-methyltransferase substrates.
3) Syntheses in the morphine alkaloid and benzomorphan fields.
4) Pattern recognition with the benzomorphans.

Previous Serial Number: Same

Principal Investigator: Arthur E. Jacobson

Other Investigators: R. Katz, S. R. Heller, R. J. Feldmann, K. Chu, H.J.C. Yeh, L. J. Sargent, E. L. May, E. Sokoloski.

Cooperating Units: NIH (DCRT: S. R. Heller, R. J. Feldmann and K. Chu; NHLI: E. Sokoloski).

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: 1) To determine the conformation of various neurotransmittors and nerve degenerating agents using the complete neglect of differential overlap (CNDO) theory.

2) To apply regression analysis to the correlation of chemical structure with the meta/para ratio of products obtained from catechol O-methyltransferase substrates.

3) To synthesize new agonist-antagonist and analgesics.

4) To apply pattern recognition in N-dimensional space to the benzomorphan analgetics.

Methods Employed: The usual organic, instrumental and computer techniques.

Major Findings: 1) Minimum energy conformations of 6-hydroxydopamine and other polyhydroxyphenethylamines, as well as dopamine and norepinephrine, have been determined. All of these compounds can exist in their gauche form with certain heteroatoms at a specific ca. 6Å distance. The molecular requirements for long-term reduction in cardiac uptake of norepinephrine have been extended using this criterion of heteroatom interatomic distance.

2) A by-product of a synthesis relating to new morphine derivatives provided a sample of a so-called "α-chloromorphide". This compound, first described in 1934, has now been found to be a 6-isochloro derivative of morphine by NMR analysis.

Significance to Biomedical Research and the Program of the Institute: The determination of physical-chemical parameters and conformations of biological molecules may give insight into the *in vivo* interactions of relatively small molecules with biological systems.

Proposed Course: A continuation and extension of the subjects listed in project titles.

Publications: Jacobson, A. E.: Structure-activity relations of analgesic and addictive potentials. *Drug Abuse*, W. Keup, Ed., C. C. Thomas, publisher, Springfield, Illinois, 1972.

Jacobson, A. E.: Narcotic Analgesics and Antagonists. *Chemical and Biological Aspects of Drug Dependence*. S. J. Mulé and H. Brill, Eds., CRC Press, Cleveland, Ohio, 1972.

Sargent, L. J. and Jacobson, A. E.: Transformations in the Morphine series. V. Reaction of codeinone with dimethyloxosulfonium methylide: structure and analgetic activity of the product and its reduced form. *J. Med. Chem.* 15: 843-845, 1972.

Jacobson, A. E., Yeh, H.J.C. and Sargent, L. J.: Nuclear magnetic resonance spectra of codeine and isocodeine derivatives. The magnetic anisotropy of a spiro oxirane ring. *Org. Magn. Resonance,* in press, 1972.

May, E. L. and Jacobson, A. E.: Perspectives in the chemistry of narcotic antagonists. *First International Conference on Narcotic Antagonists*, in press, 1973.

Katz, R. and Jacobson, A. E.: Chemical structure-activity correlation in the O-methylation of substituted catechols by catechol O-methyltransferase. *Mol. Pharmacol.* 8: 594-599, 1972.

Katz R., Jacobson, A. E., Heller, S. R. and Feldmann, R. J.: A computer system for molecular orbital calculations applied to conformational studies in dopamine. Eighth Middle Atlantic Regional Meeting of the Amer. Chem. Soc., Washington, D. C. Abstract F 4, 56, 1973.

Katz, R. and Jacobson, A. E.: A molecular orbital study of 6-hydroxydopamine and related polyhydroxyphenethylamines. Federation Proceedings, in press, 1973.

Katz, R. and Jacobson, A. E.: A molecular orbital study of 6-hydroxydopamine and related polyhydroxyphenethylamines. Mol. Pharmacol., in press, 1973.

Katz, R., Heller, S. R., and Jacobson, A. E.: A molecular orbital study of norepinephrine and dopamine: A reevaluation of structure-activity relationships in norepinephrine. Mol. Pharmacol., in press, 1973.

Heller, S. R. and Jacobson, A. E.: GINA-A graphical interactive nuclear magnetic resonance analysis program. Analytical Chemistry, 44: 2219-2222, 1972.

Serial No. NIAMDD-LC-13
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Development of Synthetic Procedures for Alkaloids

Previous Serial Number: Same

Principal Investigator: Edward M. Fry

Other Investigators: None

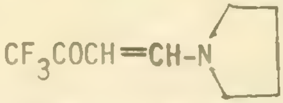
Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: The synthesis of nitrogen heterocycles.

Methods Employed: Standard methods of organic synthesis.

Major Findings: Attempted hydration of the iminium salt of 2-(1-pyrrolidinyl) propionitrile (derived from the N-oxide *via* a modified Polonovski transformation) has yielded products which are solvent dependent. Trifluoroacetic anhydride gives



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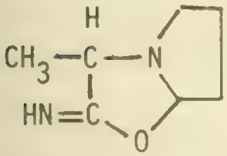
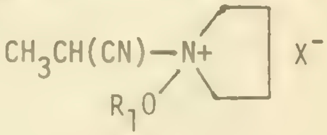


in benzene. When a more nucleophilic acid was present, the normal (N-acyl) Polonovski product was isolated. Attempts to replace the α hydrogen with hydroxyl yielded nothing useful.

Proposed Course: Other quaternary systems based on the N-oxide are being investigated. These are the related carbonic derivatives



where R=CN; CO_2Et: $R_1N=CNHR_2$. Terminated

Publications: None

Serial No. NIAMDD-LC-14
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: 6-Exo-methylene-N-cyclopropylmethyldesoxycodeine.

Previous Serial No.: Same

Prncipal Investigator: Lewis J. Sargent

Other Investigators: None

Cooperating Unit: Analytical Services Unit of this Laboratory

Man Years:
Total: 1
Professional: 1
Others: 0

Project Description:

Objectives: The replacement of the 6-carbonyl function in dihydrocodeinone by an exo-methylene group with the view to ultimately arriving at the title compound, and the evaluation of the latter as a potential narcotic antagonist.

Methods Employed: Standard organic chemical procedures including mass spectrographic and NMR techniques.

Major Findings: The Wittig reaction utilizing methylsulfinyl carbanion-dimethyl sulfoxide is known to transform carbocyclic ketones to the corresponding exo-methylene derivatives. Applying this reaction to nor-dihydrocodeinone resulted in a mixture which was separated (via column chromatography) into a small quantity of a novel (crystalline) substance of mass number 297, and a larger amount of a sirupy fraction having a mass number of 283. The difference in mass numbers of the two products suggests that the Wittig reaction apparently proceeded in two different ways. The larger mass number can be accounted for by the addition of a methylene ($=CH_2$) group to the original molecule in a novel manner. The smaller mass number fits the expected Wittig product (i.e. replacement of the 5-carbonyl function by an exo-methylene group).

Significance to Biomedical Research and the Program of the Institute: It is of interest to ascertain whether a suitably substituted desoxycodeine would show useful antagonistic properties.

Proposed Course: In order to arrive at structural proofs of the two products, further quantities will be prepared and examined by high resolution NMR.

Publications: Sargent, L. J. and Jacobson, A. E.: Reaction of codeinone with dimethyloxosulfonium methylide: Structure and analgetic activity of the product and its reduced form. J. Med. Chem. 15: 843-845, 1972.

Jacobson, A. E., Yeh, H.J.C., and Sargent, L. J.: Nuclear magnetic resonance spectra of codeine and isocodeine derivatives. Org. Magn. Resonance 4: 875-883, 1972.

Serial No. NIAMDD-LC-15
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: 1) Synthesis of N-Derivatives of Ketobemidone
2) Synthesis of N-Derivatives of (±)- and (+)-phenylmorphan
3) Synthesis of 9-Methyl-6,7-benzomorphans and their derivatives.

Previous Serial Number: NIAMDD, LC, #17 and #10

Principal Investigator: Tokuro Oh-ishi (V.S.) and E. L. May

Other Investigators: H. Ong (project #2)

Cooperating Units: Pharmacology Unit and Section on Microanalytical Services and Instrumentation of this Laboratory

Man Years:
Total: 1
Professional: 1
Other: 0.5

Project Description:

Objectives: 1) To synthesize N-derivatives (ethyl, propyl, butyl, amyl, hexyl, heptyl, allyl and cyclopropylmethyl) of ketobemidone and evaluate their analgesic and/or antagonistic activity.

2) To synthesize N-derivatives (propyl, allyl and cyclopropylmethyl) of (±)- and (+)-5-*m*-hydroxyphenylmorphan and evaluate their analgesic and/or antagonistic activity.

3) To synthesize 9-methyl-6,7-benzomorphans and derivatives by known or new routes and evaluate their analgesic activity.

Methods Employed: Reaction methods and techniques generally employed in organic chemistry.

Major Findings: 1) (a) Synthetic work has been completed and all ketobemidone derivatives described above were obtained in reasonable yields. (b) Pharmacological results. It was found that cyclopropylmethyl, allyl and propyl substituted for methyl (on nitrogen) decreased analgesic activity but did not confer antagonistic properties on the molecule in contrast to experience with systems containing 3,4,5 and 6 fused (contiguous) rings (e.g., benzomorphans, morphinans, morphines and endoethenomorphines) with a fixed axial m-hydroxy phenyl group. N-ethylnorketobemidone was inert, but appreciable analgesic activity was shown by the N-butyl compound which also possessed physical dependence capacity in monkeys. N-amylnorketobemidone is a 4 times stronger analgesic than the heroin-like ketobemidone which does not support morphine physical dependence in monkeys, in fact shows atypical properties of antagonism of long duration. The N-hexyl and N-heptyl homologs are, however, typical nalorphine-like long-acting antagonists (by the ileum and monkey, single-dose methods) with pethidine-like analgesic activity. This is a new and interesting finding.

2) (a) All phenylmorphan derivatives described above have been synthesized by custom methods, but the necessary amount to complete all pharmacological evaluation could not be obtained due to the difficulty of N-demethylation (by various methods) and insufficient starting materials. (b) Pharmacological results. Allyl, propyl and cyclopropylmethyl conferred mild antagonistic properties when substituted for methyl group of the agonist-antagonist 5-(m-hydroxyphenyl)-2-methylmorphan. These N-substituted phenylmorphans had no physical dependence property in monkeys although they had codeine-like analgesic activity.

3) A possible synthetic route to the long sought 9-methyl-6,7-benzomorphans was developed and one isomer of 2,9-dimethyl-6,7-benzomorphan was synthesized in 6-7% overall yield starting from benzylnitrile via a diastereomer of 4-dimethylamino-3-methyl-1,2,3,4-tetrahydronaphthalenone-1. The Reformatsky reaction of 5-dimethylamino-3-phenyl-2-pentanone and ethyl bromoacetate was the key step of this route. After this, standard methods were applicable. Bromination of the second possible diastereomer of the intermediate naphthalenone, on cyclization with NH_4OH did not give the benzomorphan skeleton but 4-dimethylamino-3-methyl-1-naphthol.

Reaction of 2-(p-methoxybenzyl)-3-methyl-1,2,5,6-tetrahydropyridine and 4-hydroxy-2-(p-methoxybenzyl)-3-methylpiperidine with PPA did not give the desired 9-methyl-benzomorphan.

Significance to Biomedical Research and the Program of the Institute: Constitutes a part of the overall research effort toward a chemico-pharmacological solution to the problem of narcotic addiction and toward the search for improved substitutes for morphine and codeine.

Proposed Course: To continue 3. Elaborations of each synthetic step must be required to improve the overall yield and the reaction conditions for m-hydroxyphenyl series must be established.

Publications: Ong, H. H. and May, E. L.: Phenyl-Morphan Agonists-Antagonists. J. Med. Chem. in press.

Serial No. NIAMDD-LC-16
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Molecular modification of interesting 6,7-benzomorphan as agonists and antagonists.

Previous Serial Number: NIAMDD, LC, #10

Principal Investigator: J. Harrison Ager

Other Investigators: None

Cooperating Units: Pharmacology Unit of this Section

Man Years:
Total: 0.2
Professional: 0.2
Other: 0

Project Description:

Objective: 1. Derivatives of 2'-hydroxy-2,5-dimethyl-6,7-benzomorphans. 2. Make modifications of the potent 2'-hydroxy,5,9-diethyl-6,7-benzomorphan.

Methods Employed: Standard methods of organic, physical organic and analytical chemistry.

Major Findings: 1. Acetyl, propionyl and butyryl esters of 2'-hydroxy-2,5-dimethyl-6,7-benzomorphan previously prepared in excellent yields were evaluated as the hydrochloride salts as agonists and antagonists. All three derivatives are strong agonists but also display properties of antagonism in morphine dependent monkeys (Univ. of Michigan).

2. Molecular modification of α-2'-hydroxy-5,9-diethyl-2-methyl-6,7-benzomorphan was begun; some intermediates were prepared.

This work was put aside when I was designated EEO Coordinator for NIAMDD by the Director of the Institute.

Significance to Biomedical Research and the Program of the Institute: This basic and applied research falls within the framework of this Institute and makes available new compounds of pharmacologic interest.

Proposes Course: To continue with the modification of diethyl 6,7-benzomorphans which have shown high activity as agonists and antagonists and durther examine the above-described O-acyl compounds.

Publications: None

Serial No. NIAMDD-LC-17
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Selective Modification of Free and Bound Tryptophan

Previous Serial Number: NIAMD-LC-20

Principal Investigator: Thomas F. Spande

Other Investigators: M. Jolley and B. Witkop

Cooperating Units: George Glenner, NIAMDD-LEP

Man Years:

Total: 1.0
Professional: 0.7
Others: 0.3

Project Description:

Objectives: To determine the structure(s) of the reaction products from tryptophan in peptides with the widely used histochemical reagent, Fast Red-B (FRB). To investigate the reaction of tryptophan and indole derivatives with mercuric salts.

Methods Employed: NMR, UV and IR spectroscopy, mass spectrometry and thin layer and column chromatography.

Major Findings:

a) Tryptophyl peptides with FRB (with G. Glenner). Visible spectroscopy indicates that FRB with leu-trp-leu, glu-trp-glu or gly-trp-gly at pH 3 affords a 2-azo substituted tryptophyl residue (λ_{max} = 450 nm). It was hoped that substitution of the indole ring by an electron-attracting group of roughly the same electronegativity as a nitro group might stabilize a substituted tryptophyl residue during peptide hydrolysis conditions. Unfortunately this does not seem to be the case. N-acetyl-2-(o-methoxy-p-nitrophenylazo)-tryptophan ethyl ester, a model for the 2-azosubstituted tryptophyl residue, affords as one major product in 6 N HCl hydrolysis, an uncolored, Ehrlich- and ninhydrin-negative material indicating a complex reaction has occurred. This product is not indole-3-acrylic or 3-propionic acids, nor oxindole-3-acetic or -3-propionic acids, although a UV of the hydrolysis product is compatible with an oxindole (λ_{max} = 245 nm). The other major product is surprisingly, o-methoxy-p-nitroaniline, which might be useful in quantitating reaction of tryptophan (and his or tyr?) in proteins.

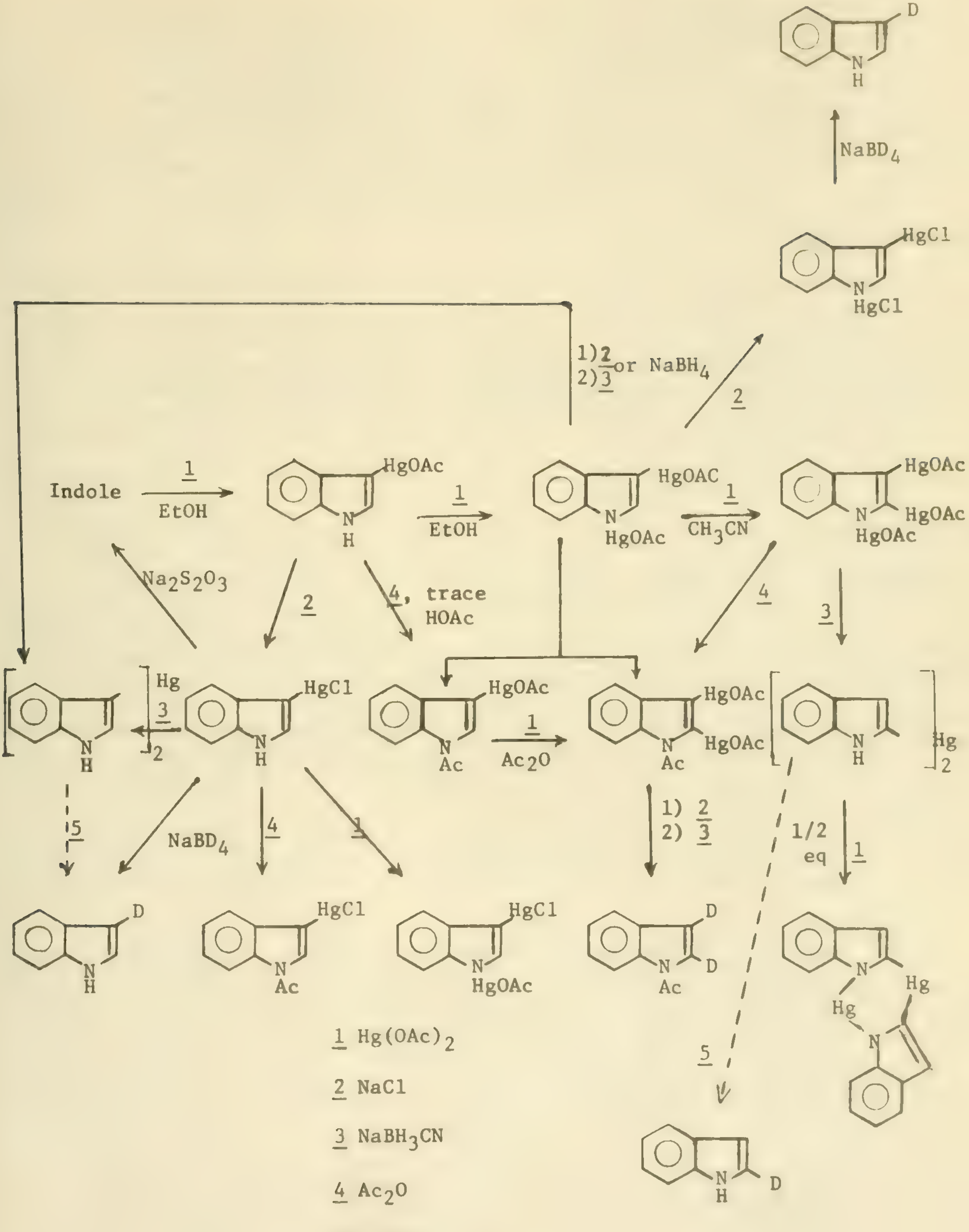
b) The reaction of tryptophan derivatives and indole with mercuric salts (in part with M. Jolley). A charge-transfer complex can be detected using UV difference spectroscopy ($\Delta\lambda_{max}$ = 297, λ_{sh} 288 nm) with tryptophan and one equivalent of mercuric ion at pH 7-8. This complex can be decomposed with a slight excess of EDTA, moderate excesses of glycine or alanine or large excesses of chloride ion as well as acidification. Similar complexes result with tryptophan methyl ester and tryptamine indicating that only the indole ethylamine grouping is involved. Indeed the complex seems specific for this structure since 2,3-dehydrotryptophan, N-acetyltryptophan, indole-3-propionic acid, N-acetyltryptamine, homotryptamine, gramine, tryptophol and skatole all fail to show UV evidence of complex formation. An attempt was made to form a complex by inter molecular interaction of a 10^3 molar excess of a glycine-mercuric ion complex and skatole but was unsuccessful. When tryptophan or tryptamine is substituted with a 5-methoxyl substituent, the difference spectrum has λ_{max} =3.5 nm. In these cases mercuration of the indole ring (presumably at the 2-position) occurs concommitant with or subsequent to complexation. Rapid ring-mercuration was observed for serotonin and bufotenin while slower mercuration was observed for metatonin and N-acetyl serotonin. 5,7-Dihydroxytryptamine was either instantaneously oxidized or mercurated by mercuric acetate.

2-Deuterated tryptophan derivative have been prepared by decomposition of 1,2-bisacetoxymercuri tryptophan esters with excess $NaBD_4$ in THF-D_2O.

c) Indole and mercuric acetate. With a view to preparing various deuterated indoles for biosynthetic or reaction mechanism studies and for possible synthetic applications (reversible blocking or activating groups, halogenination intermediates), I have effected the following conversions, most of which have not been previously reported. Deuterium incorporations of approximately 60% have been achieved though it is hoped this can be improved, working with the chloromercuri group and completely deuterated solvents. Apparently acetoxyl groups and the THF component of the aqueous (D_2O) reduction media contribute hydrogen radicals, preventing higher incorporations of deuterium.

Significance to Biomedical Research and the Program of the Institute: The stability of 2-azo substituted tryptophan derivatives to peptide hydrolysis conditions is disappointing and makes probes of tryptophan reactivity by diazonium salt coupling more difficult. We feel, however, that this should still be possible using peptide mapping techniques.

Preliminary work on the 2-deuteration of tryptophan and the preparation of 2- and 3-deuterioindole as well as 2,3-dideuterioindole is encouraging and should provide a convenient route to these materials. This route should also be adaptable to tritium labelling as well as large scale work.



1 $Hg(OAc)_2$

2 NaCl

3 $NaBH_3CN$

4 Ac_2O

5 $LiAlD_4$

It is generally thought that indoles react with mercuric salts only at a fairly acidic pH. The extreme ease with which 5-methoxy and particularly 5-hydroxy substituted tryptamines (e.g. serotonin) react with mercuric salts at physiological pH to form a fairly stable C-Hg bond is surprising. Such reactions may be of significance in the toxicology of mercuric salts and could also prove useful in the design of detoxifying drugs for mercuric ion poisoning.

Proposed Course:

To improve the deuterium incorporation into tryptophan and indole, concentrating on the chloromercuri derivatives. To study the reaction of such indole derivatives as 5-methoxy- and 5-hydroxy-thiotryptophol in their reaction with mercuric ion to see whether an intramolecular mercuration can compete with the strong mercuric ion chelation to sulfur. To extend the metallation of indoles to thallation using the relatively new reagent thallium tris-trifluoroacetate.

Honors and Awards: None

Publications:

Ottenheym, H. C. J., Spande, T. F. and Witkop, B.: The Synthesis and Reactions of a Tetrachlorodiazopiperazine. J. Org. Chem. 37: 3358, 1972.

Ottenheym, H. C. J., Spande, T. F. and Witkop, B.: Approaches to Analogs of Anhydrogliotoxin. J. Am. Chem. Soc. 95: 1989, 1973.

Spande, T. F. and Glenner, G. G.: Reaction of Indoles with a Diazonium Salt (Fast Red B). in press

Serial No. NIAMDD-LC- 18
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Synthesis and Biochemistry of Potential Antiviral and carcinostate Nucleotides and Nucleosides

Previous Serial Number:

Principal Investigator: P. Torrence and J. A. Waters

Other Investigators: B. Witkop, T. Nagamachi, Sam Baron, NIAID-LBV, C. Buckler, NIAID-LBV, H. T. Miles, LMB

Cooperating Units: Florence White, NCI-DE

Man Years:

Total: .7
Professional: .7
Others: 0

Project Description:

Objectives: The aims of this project are to investigate the chemistry and biochemistry of both normal and "fradulent" nucleosides with the objective of developing basic knowledge leading to effective anti-viral and anti-cancer agents at the monomer (nucleoside) and polymer (polynucleotide) levels.

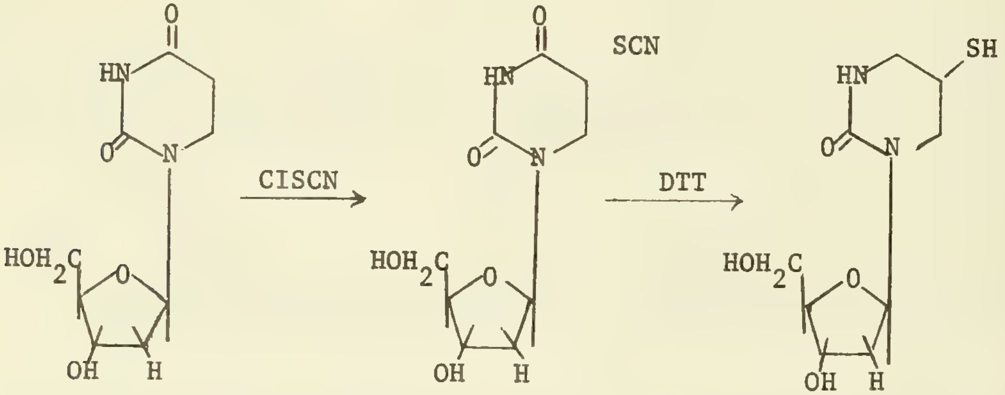
Methods Employed: Thin-layer, paper, ion-exchange and column chromatography, liquid scintillation, ultraviolet, visible, infrared, nuclear magnetic resonance, circular dichroism and mass spectroscopy.

Major Findings: We have extended physicochemical studies on the new polynucleotide poly(dU_z) [poly(2'-azido-2'-deoxyuridylic) acid]. Circular dichroism studies indicate that at 2.5°, poly(dU_z) assumes a secondary structure similar to that of poly(U) (most likely a single hairpin). Ultraviolet and CD-temperature profiles in a number of buffer systems indicate that this structure has a thermal stability which is somewhat greater than poly(U). This finding indicates that neither a 2'-OH group nor a 2'-oxygen atom is necessary for the formation of ordered structure in poly(U).

The construction of mixing curves at the isochromic wavelengths for this system revealed that poly(dU$_z$) can form both doubly- and triply-stranded complexes with poly(A), *i.e.*, poly(dU$_z$).poly(A) and 2 poly(dU$_z$). poly(A). Study of the course of thermal dissociation of these complexes appeared to indicate that both the doubly and triply-stranded complexes undergo monophasic transitions directly to constituent homopolymers (3→1 and 2→1, respectively). Unlike the previously reported A-U system and the A-rT system, no evidence fo strandwise dissociation (3→2) or rearrangement (2→3) could be found. The introduction of the azido group into the C2' position of poly(U) does not have a significant effect on the stabilities of both the triple- and double-stranded complexes when compared with the corresponding complexes in the poly(A)-poly(U) system.

In collaboration with Dr. Miles, IR melting studies on poly(A). poly(dU$_z$) are being carried out since this system may permit monitoring a helix-coil transition at a site remote from the atoms which participate in hydrogen-bonding.

We previously reported the thiocyanation of pyrimidine nucleosides by chlorothiocyanogen (ClSCN). Recent studies show that the 5-thiocyanato-pyrimidine nucleosides can be easily reduced to 5-mercaptopyrimidine nucleosides. This reaction sequence provides a quick easy synthesis of a known anti-tumor agent (5'-mercapto-2'-deoxyuridine).



We have also successfully thiocynated β-D-arabinosyluracil, proving the generality of the reaction at least for uracil derived nucleosides. This opens the way for the synthesis of a wide variety of 5-mercapto analogs. Unfortunately extensive studies have revealed that the reaction is not applicable to cytidine nucleosides.

Preliminary cytotoxicity studies on the 5-thiocyanato derivatives have revealed that 5-thiocyanato-2'-deoxyuridine (MUdR) is moderately active (2 µg/ml) uninhibiting KB cells. These studies are being extended to other cell lines.

Significance to Biomedical Research and the Program of the Institute: Fradulent nucleosides (such as MUdR) are of interest as real or potential anti-viral, immuno-suppressive or anti-tumor agents. Studies on the physical chemistry of polynucleotides aid in understanding factors important in the stability and conformation of RNA and DNA. More specifically, poly(dU_z) has been of service in understanding factors important in the induction of interferon. Such studies are useful in designing a polynucleotide complex with greater efficacy in humans than poly(I).poly(C).

Proposed Course: In collaboration with Dr. DeClercq of the Rega Institute, we shall examine the effect of poly(dU_z) on reverse transeriptase and possibly focus formation. Studies on the thiocyanto pyrimidine nucleosides will be continued here only if the additional cytotoxicity studies now in progress show sufficient promise.

Honors and Awards: None

Publications:

Torrence, P. and Witkop, B.: Enzymatic synthesis of polynucleotides containing 5,6-methylene and 5,6-dihydropyrimidines. Biochemistry 11, 1737-1745 (1972).

Torrence, P., Waters, J and Witkop, B.: Unexpected Conformational Stability of poly(2'-azido-2'-deoxyuridylic) Acid. J. Am. Chem. Soc. 94, 3638-3639 (1972).

Nagamachi, T., Torrence, P., Waters, J. and Witkop, B.: An easy access to 5-mercaptopyrimidine nucleosides through one-step synthesis of 5-thiocyanatouridine and -2'-deoxyuridine. J. Chem. Soc. Chem. Comm. 1025-1026 (1972).

Torrence, P., Bobst, A., Waters, J. and Witkop, B.: Synthesis and Characterization of Potential Interferon Inducers: Poly(2'-azido-2'-deoxyuridylic) Acid. Biochemistry, in press.

Torrence, P., Waters, J., Buckler, C. and Witkop, B.: Effect of pyrimidine and ribose modification on the antiviral activity of synthetic polynucleotides. Biochem. Biophys. Res. Comm., in press.

Serial No. NIAMDD-LC-19
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Role of Strandedness in the Induction of Interferon by Synthetic Polynucleotides

Previous Serial Number: None

Principal Investigator: Paul F. Torrence

Other Investigator: B. Witkop

Cooperating Units: E. DeClercq, Rega Institute for Medical Research, Lenven, Belgium

Man Years:

Total: .5
Professional: .5
Others: 0

Project Description:

Objectives: Any understanding of the process of interferon induction by synthetic polynculeotides must satisfactorily explain the observation that poly(A).poly(U) is 10-1000 times less active as an inducer than poly(I).poly(C). This difference has been ascribed to the rearrangement of poly(A).poly(U) to poly(A).2 poly(U) which is known to be virtually inactive. While this explanation is attractive, it is (a) difficult to rationalize in terms of the melting behavior of the poly(A).poly(U) system, and (b) it has not been experimentally verified. Alternative hypotheses may include a difference in binding of A + U versus I + C residues to a protein receptor or a recognition by the receptor of subtle conformational differences between the two systems. From a clinical viewpoint, resolution of this problem may aid in the design of inducers with greater efficacy.

Methods Employed: Thin-layer, paper, ion-exchange and column chromatography, liquid scintillation, ultraviolet, visible, infrared, nuclear magnetic resonance, circular dichroism and mass spectroscopy, ultracentrifugation.

Major Findings: If the double- to triple-strand rearrangement is the determining factor in the relative lack of activity of poly(A).

poly(U), then the synthesis of a poly(A).poly(U) analog which is incapable of triple-strand formation ought to give a complex if inducing power equal to poly(I).poly(C), all other factors being equal. Theoretically, the poly(A).2 poly(U) complex owes its existence to the formation of Hoogsteen type base pairs involving N-7 of the purine ring. Deletion of N-7 of the purine should lead to a complex with poly(U) which would be incapable of forming a triple-strand. Ikehara and Fukui reported on such a polynucleotide derived from the antibiotic tubercidin, i.e., poly(7-deaza-adenylic) acid (poly(c^7A)). There remained, however, two problems: 1) Ikehara's method of determining stoichiometry was not ideal especially in terms of what is now known and 2) the reported Tm of the poly(c^7A). poly(U (in ∿ 0.10 M NaCl) was ∿ 30°, too low to be useful in interferon studies. We reasoned that the introduction of a polarizable group (CH_3 or Br) would lead to an increase in the stability of the complex as such substituents do in the poly(A).poly(U) series. Indeed, poly(c^7A). poly(rT) and poly(c^7A).poly(br^5U) have significantly elevated Tm's of 50° and 72° respectively. All experimental evidence including mixing curves at various wavelengths (with and without Mg^{+2}), isosbestic points and melting profiles shows that only the double-stranded complexes exist in accord with theoretical expectations. The following table summarizes these findings as well as data on necessary and possibly revealing control systems. The A-U, A-rT and A-br^5U systems have been elucidated by the elegant work of Miles, Felsenfeld, Blake and others.

Tm's of Polynucleotide Duplexes in 0.15 M NaCl, 0.01 M KH_2PO_4, 0.001 M $MgCl_2$, pH = 6.95

	Tm(2→3)	Tm(3→1)	Tm(2→1)
poly(A).poly(U)	49°	62°	*
poly(A).poly(rT)	53°	76°	*
poly(A).poly(br^5U)	45°	∿ 90°	*
poly(c^7A).poly(U)	**	**	37°
poly(c^7A).poly(rT)	**	**	50°
poly(c^7A).poly(br^5U)	**	**	72°

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* Not observed under above conditions, rearrangement occurs.

** Not observed under above conditions, triple-strand incapable of existence, both experimentally and theoretically.

Poly(c^7A).poly(br^5U) meets all criteria for an effective inducer and in addition cannot form a triple-strand under any conditions. If triple-strandedness is responsible for the lack of activity of poly(A). poly(U), then poly(c^7A).poly(br^5U) ought to have activity comparable with poly(I).poly(C). If poly(c^7A).poly(br^5U) is devoid of activity, this would indicate binding of N-7 to the protein receptor or a gross conformational change induced by removal of N-7 leading to lack of receptor recognition. Intermediate activity could be rationalized on the basis of receptor affinity and/or conformational abnormalities. It seems, on the whole, that these complexes should at least reveal important information regarding the induction process. In addition, they may also reveal something of the so-called "threshhold temperature".

Significance to Biomedical Research and the Program of the Institute: For various reasons, poly(I).poly(C) does not appear to be a good inducer of interferon in humans (*e.g.* see Perspectives in Virology VII, 198-217 (1971)). The above study will lead to information to aid in the design of other polynucleotide complexes which may have greater efficacy than poly(I).poly(C).

Proposed Course: These complexes are presently being evaluated as interferon inducers by Dr. Erik DeClercq, Rega Institute for Medical Research, Leuven, Belgium. The outcome of his studies will determine the future course of this project.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-20
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Photoreactions of Nucleosides

Previous Serial Number: None

Principal Investigator: J. A. Waters

Other Investigator: B. Witkop

Cooperating Units: None

Man Years:

Total: .2
Professional: .2
Others: 0

Project Description:

Objectives: To modify nucleosides by various photochemical procedures and to evaluate the photoproducts for antitumor or antiviral activity.

Methods Employed: An aqueous solution of uridine (0.02 M) or 2',3',5'-triacetyluridine in the presence of potassium cyanide (0.2 M) was irradiated with a low-pressure mercury lamp (254 nm) for 36 hr.

Major Findings: A single, stable compound (UV_{max} 234 nm) is produced with complete disappearance of starting material. Preliminary nmr and uv data indicate a 5,6-substituted photoproduct with the -CN group at position 6.

Significance to Biomedical Research and the Program of the Institute: The cyano group imparts significant biological activity to normal nucleoside bases, *e.g.*, toyocamycin, against Ehrlich ascites carcinoma, Hela cells, *Mycobacterium tuberculosis* and others.

Proposed Course: To fully characterize the photoproduct, to elucidate the reaction mechanism and to test the compound for biological activity.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-21
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Anodic Decarboxylation of Orotidine

Previous Serial Number: None

Principal Investigator: J. A. Waters

Other Investigator: B. Witkop

Cooperating Units: None

Man Years:

Total: .2
Professional: .2
Others: 0

Project Description:

Objectives: To determine whether the anodic decarboxylation of orotidine could serve as a model for the enzymatic decarboxylation of orotidine 5'-phosphate to uridine 5'-phosphate by orotidine 5'-phosphate decarboxylase.

Methods Employed: An aqueous solution of orotidine, cyclohexylammonium salt, at basic pH was electrolyzed using platinum electrodes at 100 ma. and 10 v. for 12 hr. at 28-31°.

Major Findings: Uv kinetics show a gradual disappearance of orotidine during the electrolysis period. The formation of uridine is indicated by tlc in two solvent systems.

Significance to Biomedical Research and the Program of the Institute: Since many of the mechanistic implications of anodic decarboxylations are well established, such a model system for the biosynthesis of uridine 5'-phosphate should be an aid in the study of this particular enzyme-substrate and conceivably other decarboxylase-substrate systems.

Proposed Course: To fully characterize the anodic product(s) (uridine), to determine the reaction mechanism, and to extend the reaction to include orotidine 5'-phosphate, the true biosynthetic entity.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-22
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Poly(7-deazainosinic) Acid. A Potential Inducer of Interferon

Previous Serial Number: None

Principal Investigator: J. A. Waters

Other Investigator: B. Witkop

Cooperating Units: E. DeClercq, Katholieke Universiteit Te Leuven, Leuven, Belgium

Man Years:

Total: .2
Professional: .2
Others: 0

Project Description:

Objectives: To determine if poly(7-deazainosinic) acid, which represents a modification of the purina nucleus of the parent base, is an inducer of interferon.

Methods Employed: Synthetic 7-deazainosine 5'-diphosphate was polymerized to poly(7-deaza I) by polynucleotide phosphorylase. Melting profiles were obtained for poly(7-deaza I) and its complex with poly C.

Major Findings: Enzymatic degradations of poly(7-deaza I) were comparable to those of poly(I). The modified polymer when mixed with poly(C) formed a triple-stranded complex 2 poly(7-deaza I) . poly(C).

Significance to Biomedical Research and the Program of the Institute: Modifications of the standard synthetic polynucleotides, with regards to the alteration of the purine (pyrimidine) nucleus may provide an efficient, non-toxic inducer of interferon and may provide insight for future synthetic approaches to antiviral-antitumor drugs.

Proposed course: Poly(7-deaza I) and its complex with poly(C) are being evaluated as interferon inducers.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-23
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1972

Project Title: Photochemistry of Pharmacodynamic Amines

Previous Serial Number: NIAMD-LC-25

Principal Investigator: Bernhard Witkop

Other Investigators: None

Cooperating Units: O. Yonemitsu, Y. Kanaoka and T. Iwakuma, University of Hokkaido, Sapporo, Japan
N. Kanamaru and K. Kimura, Research Institute of Applied Electricity, Hokkaido University, Sapporo, Japan
I. L. Karle, U. S. Naval Research Lab., Washington, D.C.

Man Years:

Total: 1
Professional: .5
Others: .5

Project Description:

Objectives: Reactions for the selective modification of simple amino acids in proteins are needed for the study of the composition and function of their active sites. Although extensive interest to develop such reactions has been shown by many investigators, photochemical methods were applied only recently to this problem. Photochemical methods have been elaborated which make it possible to deliver selectively a distinct amount of energy to a system. The photochemical behavior of free and bound aromatic amino acids and the corresponding pharmacodynamic amines and certain of their derivatives therefore have been investigated.

Methods Employed: Ultraviolet irradiation, thin-layer chromatography, silica gel and ion exchange column chromatography, paper chromatography and electrophoresis, UV, IR, NMR and mass spectroscopy.

Major Findings: Irradiation of N-chloroacetyltyramine derivatives (1) give novel cage compounds (4), probably *via* intermediate 2,4-cyclohexadienones (2) and subsequent $[{}_{\pi}4_s + {}_{\pi}2_s]$ and $[{}_{\pi}2_s + {}_{\pi}2_s]$ cycloadditions.

Although cyclohexadienones easily rearrange photochemically and undergo ring opening by the addition of solvents, we failed to detect (2) as an intermediate by these reactions. However, the first direct evidence for the intermediacy of 2,4-cyclohexadienones (2) was provided by flash photolysis in the presence of dienophiles.

When a solution (0.4 mM) of N-chloroacetyltyramine (1) in ethanol-water (1:9) was flash-photolyzed at room temperature, a transient absorption at λ_{max} = 325 nm was observed, which must be due to the 2,4-cyclohexadienone (2). The decay curve followed second-order kinetics ($k = 5.3 \times 10^3$ l $mol^{-1}S^{-1}$). The relatively large rate constant indicates rapid thermal dimerization under steady-state irradiation without photolysis of the cyclohexadienone.

The second proof was chemical: When (1) was irradiated with a 10-W low pressure lamp in the presence of 2 equivalents of N-ethylmaleimide a Diels-Alder adduct was isolated in 29% yield accompanied by (4) (17%). The structure of (5) (m.p. 161-163°C) was easily confirmed by mass and NMR spectra.

The presence of (3) is suggested by analogy with the same type of cage compound obtainable by irradiation of a simple dienone dimer. In addition, we have now obtained (3) from (4) by a novel acid catalyzed reversion reaction. When (4) was digested with trifluoroacetic acid for 1 h at room temperature, (3) (m.p. 292-295°C) was isolated in 80% yield [^{1}H-NMR (D_6-DMSO, 120°C[7]): δ=5.14 (1H/s, —C=CH—), 5.94 (1H/s, —CO—C=CH—); mass: 410(M^+); UV (CH_3OH): λ_{max} = 250 nm (ε=5100)].

Serial No. NIAMDD-LC-24
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1972

Project Title: Studies on Batrachotoxin, Pumiliotoxin, Histrionicotoxin A and Other Physiologically Active Compounds from Amphibian Skins

Previous Serial Number: None

Principal Investigator: Bernard Witkop

Other Investigators: J. Daly, G. Brown, and W. Kissing

Cooperating Units: Charles W. Myers, American Museum of Natural History, New York, N. Y.
E. X. Albuquerque, Dept. of Pharmacology, University of New York at Buffalo, N. Y.
Isabella Karle, U. S. Naval Research Laboratory, Washington, D. C.
G. Habermehl, Technische Hochschule, Darmstadt, Germany
T. Tokuyama, Osaka City University, Osaka, Japan
E. J. Corey, Harvard University
H. Wehrli, ETH, Zurich, Switzerland
E. Bartels de Bernal, Universidad de Valle, Cali, Columbia
J. Gardner, D and HD:NIAMDD

Man Years:

Total: 1
Professional: .5
Other: .5

Project Description:

Objectives: To elucidate the molecular basis for the pharmacological activity of batrachotoxin, tetrodotoxin, histrionicotoxin and related substances. To isolate and elucidate the structures of other pharmacodynamically active substances found in skin extracts of neotropical frogs. To explore biosynthetic pathways involved in the formation of these unique substances.

Methods Employed: Sensitive bioassay methods, thin layer, column and gas chromatography have been used in the purification of the active principles, while NMR, mass spectroscopy, x-ray crystallography, microchemical techniques and radiochemical labeling are being utilized for structure elucidation. Isotopic tracer techniques have been used to study biosynthetic pathways. Biological activity has been investigated with both isolated enzyme systems and in intact cells.

Major Findings: Investigation of the unique pharmacological properties of batrachotoxin and various analogs has continued. The increase in sodium ion permeability normally elicited by batrachotoxin is prevented by the presence of procaine but not by other local anesthetics. Procaine, unlike another antagonist, tetrodotoxin, which merely blocks the Na^+ channels opened by the action of batrachotoxin, appears to prevent the irreversible binding of batrachotoxin to a regulatory site involved in control of Na^+ conductances. Batrachotoxin does not alter ATP levels in muscles, nor does it inhibit Na^+-K^+ ATPase activity. All analogs of batrachotoxin cause membrane depolarization by eliciting an increase in Na^+ conductance, but their relative potencies differ markedly in various biological prepartions. Batrachotoxin has no effect on a non-electrogenic cell, the human erythrocyte.

The unique properties of histrionicotoxin and various analogs has been investigated further. Perhydrohistrionicotoxin has been shown to block binding of radioactive α-bungarotoxin at a site distinct from the acetylcholine receptor site. In muscle end plates, the two binding sites for α-bungarotoxin, one blocked by d-tubocurarine and the other by perhydrohistrionicotoxin, appear to be present in a 1:1 ratio. These results in conjunction with the electrophysiological findings that histrionicotoxin and its analogs decrease K^+ conductances and independently prevent ion conductance changes due to repetitive application of acetylcholine are compatible with the hypothesis that such compounds interact not with the acetylcholine receptor, but with a site in the activated or "open" ion conductance modulator, thereby blocking ion conductance and preventing reactivation of the modulator by acetylcholine. Responsiveness is restored by dissociation of the histrionicotoxin or an analog from this macromolecule.

The structures of four more naturally occurring alkaloids related in structure to histrionicotoxin and dihydroisohistrionicotoxin have been elucidated. The compounds vary only in the presence of different hydrocarbon side chains, but the nature sf these side chains is important to the relative activity with respect to effects on K^+ conductances and at the acetylcholine receptor-ion conductance modulator complex. Identification and quantitation of histrionicotoxins, pumiliotoxins and related compounds in crude alkaloid fractions from dendrobatid frogs has been possible using chemical ionization mass spectrometry.

Significance to Biomedical Research and the Program of the Institute: The exceptionally high toxicity of batrachotoxin, its selective action on nerve preparations and its cardiotoxin properties gave the elucidation of its structure and correlations of structures with activity a high priority in biomedical research. Because of their remarkable effects on ion conductances, histrionicotoxin and various analogs provide unique tools for the study of the acetylcholine modulated ion channels in electrogenic membrane. Other compounds from frog extracts also have physiological activities which warrant their structural and pharmacological investigation. Examples of such compounds occurring in frogs and toads include the pumiliotoxins, samandarine, the bufogenins, the catecholamines, indolealkylamines and histamines, and recently hypotensive polypeptides such as bradykinin and physalaemin. Because of its structural similarity to bufotenine and lysergic acid diethylamide, a thorough investigation of the properties of dehydrobufotenine is relevant to fundamental aspects of neurochemistry.

Proposed Course of Project: The structures of various toxic alkaloids from frogs of the genera *Dendrobates*, *Colosthetus* and *Atelopus* will be elucidated. The pharmacological activity of batrachotoxin and histrionicotoxin analogs will be studied in detail. Radioactive batrachotoxin, tetrodotoxin and histrionicotoxin will be prepared and used to study their ultrastructural site of action. The unique oxidative cyclization of indolealkylamines to dehydrobufotenine will be investigated and attempts will be made to isolate the enzyme system responsible.

Honors and Awards:

Publications:

Daly, J., Albuquerque, E. X., Kauffman, F. C., and Oesch, F.: Effects of batrachotoxin on electroplax Na^+-K^+-ATPase and levels of ATP in rat muscle. *J. Neurochem.* 19: 2829-2833, 1972.

Albuquerque, E. X., Warnick, J. E., Sansone, F. M., and Daly, J. W.: The pharmacology of batrachotoxin, V. A comparative study of membrane properties and the effect of batrachotoxin on sartorius muscles of the frogs *Phyllobates Aurotaenia* and *Rana Pipiens*. *J. Pharmacol. Exp. Therap.* 184: 315-329, 1973.

Albuquerque, E. X., Barnard, E. A., Chiu, T. H., Lapa, A. J., Dolly, J. O., Jansson, S.-E., Daly, J., and Witkop, B.: Acetylcholine receptor and ion conductance modulator sites at the murine neuromuscular junction: Evidence from specific toxin reactions. *Proc. Nat. Acad. Sci. USA* 70: 949-953, 1973.

Serial No. NIAMDD-LC-25
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Service Function, Section on Microanalytical Services and Instrumentation

Previous Serial No.: NIAMDD, LC, #26

Principal Investigator: David F. Johnson

Other Investigators: P. Parisius, Byron Baer, A. Wong, W. R. Landis, H.Y.C. Yeh, N. Whittaker, B. Miller.

Cooperating Units: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are routinely determined on a quantitative basis, using ultramicro, micro, and semi-micro techniques as required. The materials analyzed include organic and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous include: mass spectrometry, infrared, nuclear magnetic resonance, ultraviolet and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colorimetric, gasometric, coulometric, potentiometric and volumetric. Assistance in the interpretation of spectra is rendered on request.

During the past year the total number of analyses performed in the microanalytical laboratory was about 13,256, a 10.5% increase over the last reporting period. A comparison of some special analyses for the two years is the following:

	1971-1972	1972-1973	% Change
Carbon	1719	2517	+46.4
Hydrogen	1719	2517	+46.4
Nitrogen	4111	7000	+70.3
Phosphorus	308	166	-46.1
Sulfur	114	78	-31.6
Halogens	198	293	+48.0
Metals	243	270	+11.1
Weight Loss	95	61	-35.8
Miscellaneous	261	354	+35.6

It is apparent that there is a substantial increase in the analysis of the most common elements performed by this service. A rough distribution of the service rendered is LC (49%), NIH, not including LC (49%), NIH not including LC (49%), and outside agencies (2%).

The NMR service includes both the Varian A60 and HA-100. The latter is used primarily as a more specialized research tool, although certain spectra are run on a service basis as the need arises. Over 800 routine spectra were run on the A60 by the principal operator, Mr. Whittaker. The instrument was also used in off hours and weekends by qualified investigators, accounting for an additional 300 spectra. An additional 673 spectra were run on a specialized service basis on the HA-100 by Dr. Yeh. Included in this figure are spectra run on the HR-220 located outsdide this Section. A significant amount of Dr. Yeh's work is done in close collaboration with other investigators and is reported elsewhere.

Routine infrared spectral analysis has picked up slightly (120 from 89). Although the least used of the instrumental services, it continues to fulfill a specialized need in the laboratory for a more detailed spectra than that obtained on the smaller infracord. Mr. Whittaker, the principal operator, has also engaged in certain specialized projects. One such project is the examination of the carbonyl and amide II bands of uracil and uracil derivatives with isotopic substitutions. Special techniques and apparatus were developed by Mr. Whittaker for this study.

A total of 1080 samples were submitted to Mr. Landis for mass spectral analysis. Each analysis involved 3 or more spectral determinations, depending on the investigators requests (high-resolution mass measurement, on-scale spectra, isotope mass-ratio measurement, fractional volatility-changing sample mixtures, molecular ion rates measurement, and standard sample spectra. Of the samples submitted, 832 were LC, 142-NIH other than LC, and 106 outside NIH (Naval Research, American Univ., and Howard Univ.).

Mr. Miller is being trained at present in the areas of gas chromatography and routine NMR on the A60. Although this new assignment requires about 50%

of his time, he continues to maintain the documentation and stocking of chemical stores.

The Section Chief continues to serve as Project Officer on Contract PH 43-65-32 between NIAMDD and the Medical Research Council of England. This contract is for the partial support of a program which provides reference steroid compounds to qualified research workers in the United States as well as to foreign investigators.

One graduate student from the Howard Univ. Medical School has been employed on a part-time basis for the past year to document the extensive steroid collection of the late Dr. Yoshio Sato. Close to 3,000 compounds have been handled and are being categorized and stored, using the Wylbur system of the NIH computer service. A number of research requests have been expeditiously handled due to ease of location by computer search. The listing, although not as yet complete, is being examined also for potential screening in the cancer program.

Publications: None.

Serial No. NIAMDD-LC-26
1. Chemistry
2. Microanalytical Services
   & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Titles: 1) Chemical structure - biological activity correlations in the enzymatic reactions of catechol-O-methyltransferase (COMT) with different substrates.
2) Molecular Orbital studies of biologically important amines.
3) Molecular Orbital studies of important biochemical intermediates (chorismic acid and derivatives).

Previous Serial No.: NIAMDD, LC, #27, 29, 31

Principal Investigator: Robert Katz

Other Investigators: A. E. Jacobson, S. R. Heller, R. J. Feldman

Cooperating Units: DCRT

Man Years:
Total: 1
Professional: 1
Other: 1

Project Description:

Objectives: Application of modern statistical and computational methods to the study of structure-activity relationship as well as chemical behavior of biologically important molecules.

Methods Employed: The Hansch approach and program for regression analysis of the physico-chemical and M.O. data assembled, and a compliation of programs for Molecular Orbital calculations using the complete neglect of differential orbitals (CNDO/2) method.

Major Findings: 1) By use of the Hansch approach a correlation of the ratio (meta:para) of O-methylated neutral side chain catechol derivatives with the steric effect of the substituent as well as the transport factor was found. Maximum reaction velocity (νmax) for the substrate enzyme reaction could be correlated with the steric and electronic parameters of the substrates. Correlations including parameters obtained through molecular orbital calculations are presently investigated.

2) Minimum energy conformations of 6-hydroxydopamine and other polyhydroxyphenethylamines, as well as dopamine and norepinephrine, have been determined. All of these compounds can exist in their gauche form with certain heteroatoms at a specific ca. 6Å distance. The molecular requirements for long-term reduction in cardiac uptake of norepinephrine have been extended using this criterion of heteroatom interatomic distance.

3) Using the CNDO/2 method on the twisted cyclohexadiene ring system, the minimum energy conformations, dipole moments and charge distributions of trans-diaxial-chorismic acid were calculated.

Significance to Biomedical Research and the Program of the Institute: Elucidation of: 1. mechanism of action of enzymes system (COMT); 2. mechanism of possible in vivo interactions between proteins and small molecules (neurotransmitters and neuro-degenerative compounds); 3. understanding of chemical and spectral behavior of important biochemical intermediates (chorismic acid and derivatives).

Proposed Course: 1) CNDO/2 calculations and regression analysis of parameters found in the different minimum energy conformations of different neutral side-chain substituted catechols with the meta/para ratios obtained in the enzymatic O-methylation will be persued.

2) Project has been terminated.

3) The trans-diequatorial-chorismic acid and both the diaxial and diequatorial isochorismic acids will be studied and results compared. The non-planar cyclohexadiene ring system will be analyzed.

Publications: Katz, R. and Jacobson, A. E.. Chemical structure-activity correlation in the O-methylation of substituted catechols by catechol O-methyltransferase. Mol. Pharmacol. 8: 594-599, 1972.

Katz, R., Jacobson, A. E., Heller, S. R., and Feldmann, R. J.: A computer system for molecular orbital calculations applied to conformational studies in dopamine. Eighth Middle Atlantic Regional Meeting of the Amer. Chem. Soc., Washington, D. C. Abstract F 4, 56, 1973.

Katz, R. and Jacobson, A. E.: A molecular orbital study of 6-hydroxydopamine and related polyhydroxyphenethylamines. Federation Proceedings, 32 (3) Abstr. 3127, 1973.

Katz, R. and Jacobson, A. E.: A molecular orbital study of 6-hydroxydopamine and related polyhydroxyphenethylamines. Mol. Pharmacol. in press, 1973.

Katz, R., Heller, S. R., and Jacobson, A. E.: A molecular orbital study of norepinephrine and dopamine: A reevaluation of structure-activity relationships in norepinephrine. Mol. Pharmacol. in press, 1973.

Serial No. NIAMDD-LC-27
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: 1) Stereochemical Course of Nucleophilic Additions to Arene Oxides. An NMR Study.
2) NMR Studies of Mono- and Di-methyl Benzene Oxide-Oxepin Valence Tautomerism.

Previous Serial No: NIAMDD, LC, 30, 32

Principal Investigator: Herman J.C. Yeh

Other Investigators: A. Jeffrey, H. Yagi and D. Jerina

Cooperating Units: None

Man Years:
Total: 1
Professional: 1
Other: 0.2

Project Description:

Objective: 1) To study the stereochemistry of reaction products resulting from action of nucleophiles upon arene oxides.

2) To study the effects of various mono- and dimethyl-substitutions on the thermodynamic and kinetic parameters of the benzene oxide-oxepin tautomerism.

Method Employed and Major Findings: 1) NMDR was used to determine the structures of reaction products. These investigations revealed stereospecificity in the nucleophilic addition to the arene oxides.

2) Variable temperature NMR spectroscopic measurements were used to determine the thermodynamic parameters and rate constants for eleven benzene oxide-oxepin systems.

Significance to Biomedical Research and the Program of the Institute: As the importance of arene oxide chemistry shown in the metabolic reaction, the present studies will provide future researchers with useful information.

Proposed Course: Various techniques of nuclear magnetic resonance will be engaged in parallel with the research development of the arene oxide series.

Publications: None

Serial No. NIAMDD-LC-28
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Steroid Transformations by Tetrahymena Pyriformis

Previous Serial No.: NIAMDD, LC, #28

Principal Investigator: Nancy S. Lamontagne

Other Investigator: David F. Johnson

Cooperating Unit: Chester E. Holmlun, Department of Chemistry,
University of Maryland, College Park, Maryland

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: The above mentioned cooperating unit has been interested in the growth inhibitory effects of steroids on the protozan Tetrahymena pyriformis. Previous studies demonstrated that a variety of steroids of mammalian origin, as well as steroid derivatives such as dialkylaminoethoxy steroids inhibit the growth of T. pyriformis, and that this growth inhibition is readily reversed by the addition of cholesterol to the medium. The mode of action of these growth inhibitory steroids is not definitely known, but inhibition of NADH oxidation and RNA synthesis, and interference with respiration and formation of lipids have been demonstrated. Of special importance in T. pyriformis metabolism is the lipid tetrahymenol, a pentacyclic triterpenoid alcohol produced in the organism by a direct, non-oxidative, proton initiated cyclization of squalene. T. pyriformis grown without added steroids produces tetrahymenol as its major unsaponifiable product. When steroids such as dehydroepiandrosterone are added to the culture an accumulation of squalene occurs with a corresponding decrease in growth rate of the organism. It has been suggested that cholesterol overcomes this inhibitory effect by performing the role (as yet unknown) of tetrahymenol in the metabolism of T. pyriformis. Since the focus of attention has always been on the action of steroids on tetrahymena, it was decided that the reverse study, i.e., the effect of tetrahymena on steroids would give additional information on the role of steroid metabolism in Tetrahymena pyriformis.

Methods Employed and Major Findings: Radioactive steroid precursers are added to T. pyriformis cultures in the exponential phase of growth. The inoculated cultures are incubated for 24 or 48 hours. Centrifugation is used to separate the cell pellet and media, which are then extracted, assayed for radioactivity, and chromatographed by column and thin-layer techniques to separate and identify the radioactive metabolic products.

All of the substrates incubated to date (androstenedione, progesterone, 17α-OH-progesterone, testosterone, and corticosterone) have yielded metabolites; as indicated by column chromatography. Preliminary thin-layer analysis indicates dehydrogenation and hydroxylation as possible transformations.

Proposed Course: Pregnenolone, estrone, and cortisone are being considered as the last steroid precursers to be incubated. Column and thin-layer analyses will be continued on completed incubation samples to identify as many of the major metabolites as possible. A large scale incubation may be done with one precurser to yield enough product for classical isolation techniques.

Publications: None

Serial No. NIAMDD-LC-29
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Stereochemical Dependence of the NMR Parameters on Geometry in ^{15}N - E and Z Aldimines.

Previous Serial No.: None

Principal Investigator: Herman J.C. Yeh

Other Investigators: Herman Ziffer and Donald Jerina

Cooperating Units: D. Boyd, Queen's University, Belfast, N. Ireland.

Man Years:
Total: 0.5
Professional: 0.5
Other: 0.5

Project Description:

Objective: To study the stereochemical dependence of the NMR parameters on the titled compounds.

Methods Employed and Major Findings: Photoirradiation and NMR tickling experiments have been employed in this work. A method for photoisomerization of these compounds has been found. Stereochemical dependence of the ^{1}H chemical shifts and coupling constants of $J_{H^{15}N}$, $J_{H^{13}C}$ and $J_{^{13}C^{15}N}$ on geometry in the titled compounds have been observed.

Significance to Biomedical Research and the Program of the Institute: Results of this study provide a better understanding of the relationship between structure, stereochemistry, and NMR parameters.

Proposed Course: Future plans include the mechanistic studies of interconversion of the C=N imine compounds and the study of Hammett relation among those compounds.

Publications: Yeh, H.J.C., Ziffer, H., Boyd, D., and Jerina, D. M.: Stereochemical dependence of the sign and magnitude of coupling constants on geometry in ^{15}N -E and Z Aldimines. J. Amer. Chem. Soc. 1973.

Serial No. NIAMDD-LC-30
1. Chemistry
2. Microanalytical Services
   & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Metabolism of C^{14}-Labelled Steroids by Adrenals from Rats with a Mammotropic Pituitary Tumor

Previous Serial No.: NIAMDD, LC, #33

Principal Investigator: David F. Johnson

Other Investigator: Nancy Lamontagne

Cooperating Unit: Robert W. Bates (NIAMDD-LNE)

Man Years:
- Total: 1
- Professional: 1
- Other: 0

Project Description:

Objective: Quantitative and kinetic study of steroid biogenesis in rat adrenals subjected to long-term stress by ACTH, growth hormone, and prolactin.

Methods Employed: Radioactive C^{14}-progesterone and C^{14}-pregnenolone were incubated with normal rat adrenals and adrenals from rats subjected to long-term stress of tropic hormones of the anterior pituitary. Substrates were incubated from varying lengths of time (20-120 minutes). Extracts of the incubates were chromatographed on silicic acid columns by a technique developed in this laboratory which allows for separation of adrenocortical and keto-steroids on a single column. Zones of radioactivity from column chromatography are then further characterized and purified by thin-layer techniques. Endogenous and C^{14} metabolites are calculated on the basis of C^{14}/H^3 ratios, using appropriate tritiated standards.

Major Findings: Earlier studies in this laboratory demonstrated greater conversion of progesterone by normal rat adrenals than with the adrenal tissue from tumor animals. Quatitative differences in the metabolic products was also found in the two groups. Most recent results show that the conversion of pregnenolone to progesterone in the adrenals of the tumor animals is markedly reduced. Isolation of metabolites from pregnenolone incubations further indicates a by-pass biosynthetic pathway than that of normal rat adrenals.

Significance to Biomedical Research and the Program of the Institute: The tumor-bearing rats show lowered blood cholesterol and splanchnomegaly of the liver, kidney, and heart. The fact that splanchnomegaly does not occur in adrenalectomized rats with MtT, and administration of adrenal steroids to adrenalectomized MtT rats did not exhibit splanchnomegaly, suggested that some unknown factor of adrenal origin was the possible causative agent. Present work is designed to investigate the origin of this factor.

Proposed Course: Continuation of studies to elucidate the biosynthetic pathway exhibited by adrenal tissue from MtT rats, using pregnenolone as substrate, and identification of metabolites produced.

Publications: None

Serial No. NIAMDD-LC-31
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The study of the Constituents of Solanum Xanthocarpum

Previous Serial No.: NIAMDD, LC, #37

Principal Investigator: Genjiro Kusano and Robert Katz

Other Investigators: Yoshio Sato and David F. Johnson

Cooperating Unit: Malcolm J. Thompson and William E. Robbins, Insect Physiology Laboratory, Agricultural Research Service, U. S. Department of Aglriculture, Beltsville, Maryland

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: To isolate and identify the highly toxic component effecting the housefly larvae found in the extracts of Solanum xanthocarpum.

Methods Employed and Major Findings: It was established that two components of the Solanum xanthocarpum extract, solamargine and solasonine in mixture, have a synergistic, highly toxic effect on the house fly larvae.

Proposed Course: A quantitative bioassay is attempted in order to establish the optimum ratio of solamargine and solasonine for synergistic activity.

Publications: None

Serial No. NIAMDD-LC-32
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Role of Metal Ions in Histidine Ammonialyase.
A ^{35}Cl NMR Study.

Previous Serial No.: None

Principal Investigator: Herman J.C. Yeh

Other Investigators: Claude B. Klee, Kenneth Kirk and Louis A. Cohen

Cooperating Units: None

Man Years
Total: 0.2
Professional: 0.2
Other: 0

Project Description:

Objective: The role of metal ions in the activity of histidine ammonia-lyase from Pseudomonas has been the subject of conflicting reports. It has been known that the ^{35}Cl NMR technique is capable of elucidating the binding of metal ions to the enzyme. An investigation of the metal-enzyme interaction by ^{35}Cl NMR is suggested.

Methods Employed and Major Findings: ^{35}Cl Nuclear magnetic resonance will be employed for the study of the metal-enzyme interaction.

Significance to Biomedical Research and the Program of the Institute: Results of this study will permit more thorough interpretation of the role of metal ions in the activity of the titled enzyme.

Proposed Course: A successful ^{35}Cl NMR examination of this system will extend the application of the ^{35}Cl NMR to other enzyme systems.

Publications: None

Serial No. NIAMDD-LC-33
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: 1) ^1H and ^{19}F NMR Studies of Ring Flourinated Imidazole Derivatives. 2) NMR Studies of Intramolecular Interaction of (5-methylimidazole-4 yl) Dimethyl Butyric Acid and Its Analogs.

Previous Serial No.: NIAMDD, LC, #36, 52

Principal Investigator: Herman Yeh

Other Investigaors: K. Kirk and L. Cohen (project I); Y. Kikugawa and L. Cohen (project 2).

Cooperating Units: J. Cohen (DCRT)

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: 1) To study the chemico-physical properties of the ring flourinated imidazole derivatives and their interaction with enzymes. 2) To study the role of imidazole side chain in a catalytic action of esterases.

Methods Employed and Major Findings: 1) ^1H and ^{19}F NMR titration experiments were used to examine 2-fluoroimidazole, 2-fluoro-histidine, 4-fluoro-imidazole and 4-fluoro-histidine. pKa's of these four compounds have been determined. The effects of neighboring charge groups on the ring ^{19}F chemical shifts were found to be significant, but not on the ^1H chemical shifts.

2) ^1H NMR titration experiment was used to examine (5-methylimidazole-4 yl) dimethyl butyric acid and its analogs. pKa's of nine compounds were determined. The intramolecular effect of the carboxyl group to the imidazole group was found significant in several compounds.

Significance to Biomedical Research and the Program of the Institute: Results of physical-chemical properties on these compounds will permit more thorough interpretation of the role of imidazole group in biological function.

Proposed Course: NMR examinations of ring flourinated imidazole derivatives and (5-methylimidazole-4 yl) alkyl acids have been completed. Further studies of the interactions of these compounds with enzymes are being planned.

Publications: None

Serial No. NIAMDD-LC-34
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies of Human Erythrocytes and Frog Muscle by ^{23}Na NMR.

Previous Serial No.: None

Principal Investigator: Herman J.C. Yeh

Other Investigators: E. D. Becker

Cooperating Units: F. J. Brinley, Jr., Johns Hopkins Medical School
Baltimore, Md.

Man Years:
Total: 1
Professional: 1
Other: 0.2

Project Description:

Objective: To study the physical state of water and ions, particularly sodium, in a variety of cells or tissues.

Methods Employed and Major Findings: ^{23}Na NMR has been used to study the physical state of sodium in frog muscle and human erythrocytes. NMR spectra of fresh intact muscle revealed a single absorption band with a width of about 38 Hz equivalent in intensity to about 53% of the total muscle sodium. The intensity of the sodium signal in fresh cells was 98% of that present in the same samples after complete hemolysis of the cells. The present data provide no indication that strong interaction of the bulk of the intracellular sodium ions with intracellular constituents is necessary to account for such transport phenomena as exclusion of sodium from the cell interior or regulation of sodium entry and exit in human erythrocytes.

Significance to Biomedical Research and the Program of the Institute: An understanding of the binding and transport of sodium is basic to electrophysiology. These studies will assist in distinguishing between several hypotheses for the transport of sodium across cell membranes.

Proposed Course: ^{23}Na NMR is planned for the study of intracellular sodium in human sickle cells.

Publication: Yeh, H.J.C., Brinley, Jr., F. J., and Becker, E. D.: Nuclear Magnetic Resonance studies on intracellular sodium in human erythrocytes and frog muscle. Biophysical Journal 13: 56-71, 1973.

Serial No. NIAMDD-LC-35
1. Chemistry
2. Microanalytical Services
   & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Study of the Photochemical Reactions of Certain Derivatives of Indole and Related Heterocyclic and Carbocyclic Compounds.

Previous Serial No.: NIAMDD, LC, #45

Principal Investigator: Calvin M. Foltz

Other Investigators: None

Cooperating Units: None
Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: To develop organic chemical and photochemical methods of utility in the study of biological and medical problems and to synthesize organic compounds of biological and medical interest. To study the mechanism of the photocyclodehydrohalogenation reaction produced by uv irradiation of N-chloroacetyltryptamine and related compounds and to study the scope of its applicability to other heterocyclic and non-heterocyclic systems. To study the scope and mechanism of the rearrangement which has been found to take place on uv irradiation of 3-(3'-chloro-2'-oxopropyl)indoline-2-one. To study the photo-Fries rearrangement of esters, particularly of halo-acids, with a view to developing methods of modifying and synthesizing compounds of potential biological and medical interest.

Methods Employed: Standard organic chemical techniques; irradiation of solutions of organic compounds with ultraviolet radiation generated by mercury-vapor lamps; column, gas, and thin layer chromatography; and ultraviolet, visible, infrared, nuclear magnetic resonance, and mass spectroscopy.

Major Findings: Photolysis of N-chloroacetyltryptamine, N-chloroacetyl-3-(3'-aminopropyl)-indole, and N-3-(4'-aminobutyl)indole with mercury-vapor lamps results in the formation of high-melting dehydrohalogenation products in yields of 50%, 30%, and 20% respectively. The product of the photodehydrohalogenation of N-chloroacetyltryptamine is the tricyclic lactam, 6-oxo-3,4,6,7-tetrahydro-1H,5H-azocin[4,5,6-c,d]indole, which results from cyclization in the 4-position of the indole moiety. Reduction of the lactam with diborane yielded the amine, 3,4,6,7-tetrahydro-1H,5H-azocin-[4,5,6-c,d]indole. Nuclear magnetic resonance spectroscopy of the amine in chloroform solution has provided conclusive evidence that the photocyclization, which could have taken place at the 2- or the 4-position of the indole moiety, did not take place at the 2-position; spin decoupling experiments established the existence of the proton in the 2-position. The tricyclic amine is also of interest because of its potential physiological activity.

The photolysis products of N-chloroacetyl-3-(3'-aminopropyl)indole and N-chloroacetyl-3-(4'-aminobutyl)indole are thought to be the tricycliclactams, 7-oxo-3,4,5,6,7,8-hexahydro-1H-azonin[6,5,4-c,d]indole and 8-oxo-3,4,5,6,8,9-hexahydro-1H,7H-azecin[6,5,4-c,d]indole, respectively. It is expected that nmr spectroscopy of the amines derived from them will likewise provide the final evidence needed to define their structures. Diborane reduction of the photo product from N-chloroacetyl-3-(3'-aminopropyl)indole so far has yielded mixtures from which the amine has not yet been isolated. These amines will also be of pharmacological interest.

As part of the study of this photodehydrohalogenation reaction N-chloroacetyl-2-(α-naphthyl)ethylamine was prepared as a prototype of polycyclic aromatic systems. Irradiation with a mercury-vapor lamp resulted in photocyclization with the formation of the tricyclic lactam, 2-oxo-2,3,4,5-tetrahydro-1H-naphth[1,8-de]azocine, in 47% yield. N-Choroacetyl derivatives of compounds such as aniline, benzylamine, β-phenylethylamine, 1-aminomethylnaphthalene, and α- and β-naphthylamines also have been prepared and phototitrations carried out. The utility of the automatic phototitation technique was discovered in the course of study of photodehydrohalogenations and already has been applied to several series of compounds. The kinetic data obtained in this way provides useful information on the course and mechanism of such reactions. Further refinements in the technique to extend its utility are being made. Several years ago in the course of studies of intermolecular photodehydrohalogenation reactons, anomalous phototitration results were obtained with phenyl chloroacetate as one of the reactants. A literature search revealed that phenyl chloroacetate apparently undergoes a photo-induced Fries rearrangement followed by a cyclodehydrohalogenation reaction. These reactions may afford a mild specific method for modifying certain compounds of biological or medical interest or for synthesizing compounds of special interest. Studies of this reaction are in progress.

Significance to Biomedical Research and the Program of the Institute: New compounds produced in this work are of biological and medical interest. The organic chemical and photochemical reactions being developed can be applied to the synthesis of other compounds of biological and medical interest, to the modification of compounds of biological and medical interest and to the development of methods for use in the study of biological and medical problems.

Proposed Course: To continue to study the mechanisms of the reactions described in this report and to determine the extent to which they can be applied to related compounds, to related types of compounds and particularly to compounds of biological interest. To apply the methods developed to the synthesis of compounds of particular biological or medical interest. To develop and study other photochemical and organic chemical reactions and techniques with potential utility for the synthesis, modification, characterization, and study of molecules of biological and medical interest. To develop chemical methods which can be applied in the study of biological and medical problems.

Publications: None

Serial No. NIAMDD-LC-36
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Nuclear Magnetic Resonance Spectra of Codeine and Isocodeine Derivatives.

Previous Serial No.: NIAMDD, LC, #51

Principal Investigator: Herman J.C. Yeh

Other Investigators: Arthur Jacobson and Lewis J. Sargent

Cooperating Units: None

Man Years:
Total: 0.5
Professional: 0.5
Other: 0

Project Description:

Objective: To determine the structures of spiro oxirane (1) derivatives of codeinone, codeine (2), isocodeine (3), 6-methylcodeine (4), and 6-methylisocodeine (5), and to study the effect of a spiro oxirane ring on the ^1H chemical shift and coupling constant.

Methods Employed and Major Findings: NOE and double resonance experiments have been employed to confirm the conformation of 1, and the configuration about C-6 of 6-methylcodeine and 6-methylisocodeine. An interchange of the chemical shifts of the olefinic protons in 1 was noted, as compared with those in all of the other compounds, This interchange could be attributed to the bond anisotropies of the oxirane moiety.

Significance to Biomedical Research and the Program of the Institute: These studies are important in the determination of absolute configurational assignments for codeine and its derivatives which are widely used as an analgesic drug.

Proposed Course: This work has been completed.

Publications: Jacobson, A. E., Yeh, H.J.C., and Sargent, L.J.: Nuclear Magnetic Resonance Spectra of Codeine and Isocodeine Derivatives. The Magnetic Anisotropy of a Spiro Oxirane Ring. Org. Magnetic Resonance 4: 875-883, 1972.

Serial NO. NIAMDD-LC-37
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Modifications of the HA-100 Probe for $\{X\}$ - ${}^{1}H$ Nuclear Magnetic Double Resonance

Previous Serial No.: None

Principal Investigator: Herman J.C. Yeh

Other Investigators: None

Cooperating Units: R. Tschudin (Varian Engineer); D. Lincoln and E. Lustig (FDA).

Man Years:
Total: 0.5
Professional: 0.5
Other: 0

Project Description:

Objective: In many NMR studies, it is often found that a heteronuclear magnetic double resonance (NMDR) experiment is essential in order to obtain needed information. Our Varian HA-100 spectrometer was not equipped for such NMDR experiment. A HA-100 probe modification was, therefore, initiated. The aim of this work was to develop a modified probe that will, one, not affect the normal NMR experiment; two, offer a significant decrease in the time required to conduct the double resonance experiments; three, offer versatile $\{X\}$-${}^{1}H$ NMDR, e.g., $\{X\}$= ${}^{2}H$, ${}^{13}C$, ${}^{14}N$, ${}^{15}N$ and ${}^{31}P$, and four, permit efficient continuous wave decoupling.

Methods Employed and Major Findings: Circuit and mechanical modification of the probe were designed and constructed. The modified spectrometer has met the main goal of the modification. Major Finding: for an example see project number #29.

Significance to Biomedical Research and the Program of the Institute: The modified spectrometer is now available to investigators at the Institutes who require NMDR studies.

Proposed Course: The project has been completed.

Publications: Yeh, H.J.C., Tschudin, R. G., Lincoln, D. N. and Lustig, E.: Modification of the HA-100 probe for $\{\{X\}\}-{}^{1}H$ nuclear magnetic double resonance. J. Magn. Resonance, in press 1973.

Serial No. NIAMDD-LC-38
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: $\{^{31}P\}$-^1H Nuclear Magnetic Resonance Studies of Pyridoxal Phosphates and Derivatives.

Previous Serial No.: None

Principal Investigator: Robert Katz

Other Investigators: Herman J. Yeh, David F. Johnson

Cooperating Units: None

Man Years:
Total: 0.5
Professional: 1
Other: 0

Project Description:

Objective: Conformational studies of the title compounds and their interactions with macromolecules.

Methods Employed and Major Findings: A modified Varian HA-100 NMR spectrometer which enables studies of ^{31}P and ^1H chemical shifts by double resonance technique is being used. Titrations of Vit B_6 phosphates are performed and pKa of the different structural groups established. Studies are in the preliminary stage and results are not yet available.

Significance to Biomedical Research and the Program of the Institute: Elucidation of the role of the phosphoric acid group in biologically important compounds and its interaction with proteins.

Proposed Course: Possible interaction between above substrates and different amino acids, peptides and proteins will be studied.

Publications: None.

Serial No. NIAMDD-LC-39
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Structure Determination of Tomatillidine

Previous Serial No.: NIAMDD, LC, #29

Principal Investigator: Genjiro Kusano

Other Investigators: David F. Johnson and Herman Yeh

Cooperating Units: None

Man Years:
Total: 1
Professional: 1
Other: 0.5

Project Description:

Objective: In earlier investigations of dihydrotomatillidine (I) and 24-oxo-solacongestidine in this laboratory, it was found that an α,β-unsaturated keto derivative (III) derived from a $\Delta^{22(N)}$22,26-imino compound (II) isomerized to another compound (IV) on silica gel thin-layer chromatographic plates. From the spectroscopic data of IV and some consideration on the structure of III some questions were raised concerning the assigned structures of tomatillidine, dihydrotomatillidine, and 24-oxo-solacongestidine. Studies were carried out to elucidate these structures by NMR and synthesis by unambiguous routes.

Methods Employed and Major Findings: A pseudo compound, 3,16-di-O-acetyl pseudosolasodine (II), prepared from solasodine by treatment with acetic acid-acetic anhydride-zinic chloride, was oxidized with SeO_2 in dioxane. This reaction seemed to afford one major product (III) with traces of another (IV) on a TLC plate. Separation on a preparative TLC gave two products in a 1:1 ratio, suggesting change of one product to the other on the plate. Oxidation of II with active MnO_2 in $CHCl_3$, in which the major 23-oxo-compound (III) is expected, yielded instead a 60% yield of the isomeric compound IV. NMR and synthetic studies of the various compounds indicate a new assignment for the structure of tomatillidine.

Significance to Biomedical Research and the Program of the Institute: Elucidation of the true structure of tomatillidine can lead to a better understanding of the role of this alkaloid in plant biosynthesis.

Proposed Course: Additional experiments will be carried out to support the proposed structure assignment.

Publications: None

Serial No. NIAMDD-LC-40
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Metabolism of C^{14}-Labelled Steroids by Adrenals from Pseudohermaphrodite Rats

Previous Serial No.: Same

Principal Investigator: David F. Johnson

Other Investigator: Nancy Lamontagne

Cooperating Unit: C. Wayne Bardin, formerly Endocrinology, NCI; present Pennsylvania State Medical School, Hershey, Penn.

Man Years:
Total: 0
Professional: 0
Other: 0

Project Description:

This project is terminated.

Serial No. NIAMDD-LC-41
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Pyramidal Isomerism of Solasodine and Its Derivatives

Previous Serial No.: Same

Principal Investigator: Genjiro Kusano

Other Investigators: Herman J.C. Yeh and Yoshio Sato (deceased)

Cooperating Unit: None

Man Years:
Total: 0
Professional: 0
Other: 0

Project Description:

This project is terminated.

Serial No. NIAMDD-LC -42
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30 1973

Project Title: Toxicity Studies of Solanum Bulbocastanum

Previous Serial No.: Same

Principal Investigator: David F. Johnson

Other Investigators: Yoshio Sato (deceased)

Cooperating Unit: Potato Introduction Project
USDA Agricultural Research Service
Department of Horticulture
University of Wisconsin
Madison, Wisconsin

Man Years:
Total: 1
Professional: 1
Other: 1

Project Description:

Previous investigations resulted in isolation of an extract from processing this plant which was indicative of a toxic principal in the alkaloid fraction. Subsequent attempts at isolation of this material with plants grown during a growing season quite unlike the original gave negative results. A third attempt, recently completed, with plants grown under conditions similar to the first were also negative. No further attempt will be made to pursue this problem. This project is terminated.

Serial No. NIAMDD-LC-43
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biosynthesis of Stigmasten-3β-ol and 3',5'-Cyclic AMP by the Slime Mold (Dictyostelium discoideum)

Previous Serial No.: Same

Principal Investigator: David F. Johnson

Other Investigators: None

Cooperating Unit: Micah Krichensky, (D:IR)

Man Years:
Total: 0
Professional: 0
Other: 0

Project Description:

This project is terminated.

Serial No. NIAMDD-LC-44
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: A synthesis of ecdysone-like compounds from carpesterol.

Previous Serial No.: NIAMDD, LC, #53

Principal Investigator: Genjiro Kusano

Other Investigator: Yoshio Sato (deceased)

Cooperating Unit: William E. Robbins
Insect Physiology Laboratory
Agricultural Research Service
U. S. Department of Agriculture
Beltsville, Maryland 20705

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

This project is terminated.

Serial No. NIAMDD-LC-45
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: A Synthesis of Tomatillidine from Solasodine

Previous Serial No.: NIAMDD, LC, #35

Principal Investigator: Genjiro Kusano

Other Investigator: Yoshio Sato (deceased)

Cooperating Unit: None

Man Years:
Total: 0
Professional: 0
Other: 0

Project Description:

This project is terminated.

Serial No. NIAMDD-LC- 46
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Synthesis of Solaphyllidine

Previous Serial No.: NIAMDD, LC, #38

Principal Investigator: Genjiro Kusano

Other Investigator: Yoshio Sato (deceased)

Cooperating Units: None

Man Years:
Total: 0
Professional: 0
Other: 0

Project Description:

This project is terminated.

Serial No. NIAMDD-LC-47
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Role of Cyclic Adenosine Monophosphate in the Central Nervous System.

Previous Serial Number: NIAMDD-LC-55

Principal Investigator: John Daly

Other Investigators: M. Huang, P. Skolnick

Cooperating Units: F. Bloom and B. Hoffer, St. Elizabeths Hospital, NIMH
E. Gruenstein, M.I.T., Cambridge, Massachusetts
H. Shimizu, Nippon Roche Research Center, Kanagawa, Japan
J. Schultz, Guest Worker, LC-NIAMDD and University of Tuebingen, Germany

Man Years

Total: 3.6
Professional: 2.3
Other: 1.3

Project Description:

Objectives: To determine the factors that govern the formation and metabolism of cyclic AMP in the central nervous system. To investigate the possible role of cyclic AMP in regulating biochemical reactions and membrane properties in brain tissue.

Methods Employed: Isotopically labeled compounds have been used to study the formation and breakdown of adenine nucleotides in brain slices, and with partially purified enzymes.

Major Findings: The effects of various agents on levels of ATP, ADP, AMP and cyclic AMP in slices of guinea pig cerebral cortical grey matter have been determined. Levels of radioactive adenine nucleotides, labeled by a prior incubation with radioactive adenine were also determined. The results indicate that adenine is incorporated into morphological compartments containing adenine nucleotides that serve as the major source of nucleotide precursor for cyclic AMP. The nucleotides of this compartment represent less than one third of the total adenine nucleotides of the slice. The cyclic AMP that accumulates in labeled guinea pig cortical slices after stimulation with a variety of agents has approximately the same specific activity suggesting that all of the agents interact with similar compartments of labeled nucleotides. However, an initial transient increase in cyclic AMP levels elicited by histamine appears to take place in a compartment, whose adenine nucleotides are labeled to a much lower extent with radioactive adenine. Pulse labeling techniques with radioactive adenine reveal a high turnover rate for cyclic AMP which is proportional to the magnitude of the steady state level of the cyclic nucleotide. Adenosine stimulates accumulation of cyclic AMP and potentiates or reveals the stimulatory effect of biogenic amines. These effects on cyclic AMP accumulation are not potentiated by phosphodiesterase inhibitors that are quite effective in the absence of adenosine. Biogenic amines can elicit only one accumulation of cyclic AMP in brain slices, but combinations of adenosine and the biogenic amine are effective during a sequence of at least four restimulations. The specific activity of cyclic AMP in adenine labeled slices remains nearly constant during these sequential stimulations indicating that under conditions of repetitive conversions of at least 40% of the compartmentalized adenine nucleotides to cyclic AMP, significant translocation of nucleotides from less highly labeled compartments does not occur. An intermediate incubation of amine-stimulated slices with adenosine does not restore responsiveness to an amine. However, the presence of a phosphodiesterase inhibitor does partially restore amine-elicited accumulations of cyclic AMP during a second stimulation.

Responses of the cyclic AMP-generating system to biogenic amines are mediated *via* separate receptors for catecholamines, histamine and serotonin. In guinea pig cerebral cortical slices, a preponderance of α-adrenergic stimulatory receptors are present, while in rat both α- and β-receptors are present. In mouse primarily β-receptors are involved in catecholamine-mediated increases in cyclic AMP levels. Pretreatment of rats with 6-hydroxydopamine and a related substance, 2,3,5-trihydroxyphenethylamine, cause an increase in the maximal accumulation of cyclic AMP elicited by catecholamines, but not by other agents. Both α- and β-adrenergic receptor-mediated increases in cyclic AMP levels were potentiated by this pretreatment. Dihydroxytryptamines did not significantly alter the magnitude of cyclic AMP accumulations elicited in rat cortical slices by a variety of agents including serotonin.

Norepinephrine, serotonin and adenosine elicit accumulations of cyclic AMP in slices prepared from different neocortical areas and from limbic cortex of squirrel monkey. The extent of the accumulation of cyclic AMP differs among the various regions studied.

The accumulation of radioactive cyclic AMP in cortical slices, prelabeled with radioactive adenine is greater in the parent strain of mice from which the quaking mouse mutation was obtained. The lower accumulations of radioactive cyclic AMP in the quaking mouse appear to be due to alterations in the extent of incorporation of radioactive adenine into compartments whose nucleotides serve as precursors of cyclic AMP, rather than a result of a lower activity of the cyclic AMP-generating system. The specific activity of the labeled nucleotides that serve as precursors for cyclic AMP during stimulation by either norepinephrine, veratridine, or adenosine are not identical in mouse cortical slices labeled during a prior incubation with radioactive adenine.

Significance to Biomedical Research and the Program of the Institute: The key role of adenyl cyclase and cyclic AMP in regulating cellular activity in response to external stimuli in many biological systems make elucidation of their role in brain of fundamental importance to an understanding of function in this organ.

Proposed Course of Project: The morphological location of the labeled pools of adenine nucleotides will be determined. The role of phosphoribosyl transferase and adenosine kinease in maintaining this pool will be explored. Secondary effects due to elevated cyclic AMP levels in brain tissue will be investigated. The *in vivo* effects of drugs on the responsiveness of the cyclic AMP system will be explored. Attempts to label the precursor pool with fluorescent and radioactive adenine analogs will be made. The nature of the adenosine receptor will be studied further using adenosine analogs modified for possible affinity labeling.

Honors and awards: None

Publications:

Schultz, J. and Daly, J. W.: Cyclic adenosine 3',5'-monophosphate in guinea pig cerebral cortical slices. I. Formation of cyclic adenosine 3',5'-monophophate from endogenous adenosine triphosphate and from radioactive adenosine triphosphate formed during a prior incubation with radioactive adenine. *J. Biol. Chem.* 248: 843-852, 1973.

Schultz, J. and Daly, J. W.: Cyclic adenosine 3',5'-monpphosphate in guinea pig cerebral cortical slices. II. The role of phosphodiesterase activity in the regulation of levels of cyclic adenosine 3',5'-monophosphate. *J. Biol. Chem.* 248: 853-859, 1973.

Schultz, J. and Daly, J. W.: Cyclic adenosine 3',5'-monophosphate in guinea pig cerebral cortical slices. III. Formation, degradation and reformation of cyclic adenosine 3,5-monophosphate during sequential stimulations by biogenic amines and adenosine. *J. Biol. Chem.* 248: 860-866, 1973.

Shimizu, H., Takenoshita, M., Huang, M., and Daly, J. W.: Accumulations of adenosine 3',5'-monophosphate in brain slices: Interaction of local anaesthetics and depolarizing agents. *J. Neurochem.* 20: 91-95, 1973.

Schultz, J. and Daly, J. W.: Adenosine 3',5'-monophosphate in guinea pig cerebral cortical slices: Effects of α- and β-adrenergic agents, histamine, serotonin and adenosine. *J. Neurochem.* In press.

Skolnick, P., Huang, M., Daly, J., and Hoffer, B.: Accumulation of adenosine 3',5'-monophosphate in brain slices from discrete regions of squirrel monkey cerebral cortex: Effect of norepinephrine, serotonin and adenosine. *J. Neurochem.* In press.

Serial No. NIAMDD-LC-48
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Mechanism of Enzymatic Oxidation and the Role of Reactive Intermediates in Drug Metabolism, Hepatotoxicity and Carcinogenesis

Previous Serial Number: NIAMDD-LC-56

Principal Investigator: Donald M. Jerina

Other Investigators: J. Daly, H. Yagi, J. Tomaszewski, H. Selander, H. Yeh

Cooperating Units: D. Boyd, Univ. Belfast, Belfast, Ireland
H. Ziffer, NIAMDD:LPB
D. Reed, Oregon State Univ., Corvallis, Oregon
O. Chapman, Iowa State Univ., Ames, Iowa
J. Rice, NCI:ET
A. Y. H. Lu, A. Conney, and W. Levin, Hoffmann-LaRoche, Nutley, New Jersey
D. W. Nebert, NICHHD:LBS
J. P. Ferris, Rensselaer Institute, Troy, New York
T. C. Bruice, Univ. of California, Santa Barbara, Calif.
D. T. Gibson, Univ. of Texas, Austin, Texas
G. Berchtold, M.I.T., Cambridge, Massachusetts
E. D. Becker, NIAMDD:LPB
F. McLafferty, Cornell Univ., Ithaca, New York
P. Dansette, Guest Worker, NIAMDD:LC
A. Jeffrey, NIAMDD:LBP
S. Milstien, NIMH:LNC
J. A. Dipaolo, NCI:BGY

Man Years

Total: 4.6
Professional: 4.3
Other: 0.3

Project Description:

Objectives: To study the basic mechanism of oxygen activation and enzymatic incorporation into organic substrates. To explore enzymatic and non-enzymatic reactions of labile intermediates such as epoxides and cyclic peroxides. To devise methods for the synthesis and study of the chemical reactions of arene oxides. To investigate the significance of labile oxygenated intermediates to the cytotoxicity and carcinogenicity of xenobiotic substances.

Methods Employed: Isotopically labeled substrates, water and oxygen gas, have been to study the oxidation of organic compounds with a variety of enzymes and the subsequent metabolism of labile intermediates. Nuclear magnetic resonance spectroscopy, mass spectrometry, x-ray crystalography, and other physico-chemical techniques have been employed frequently in these studies. In addition to the usual techniques employed for the separation of metabolites, high pressure liquid chromatography is being pioneered in this area.

Major Findings: Efforts to establish practical synthetic methods for the preparation of labile arene oxides have proved most rewarding. In addition to adapting known synthetic methods for the elaboration of all the mono- and dimethylbenzene oxides as well as the oxide of 1,3,5-trimethylbenzene, an entirely new method has been developed which employs a latent epoxide precursor. The method is proving extremely adaptable to the synthesis of hithertofore inaccessible arene oxides -- particularly those of the polycyclic, carcinogenic hydrocarbons.

Detailed examination of the mechanisms by which arene oxides rearrange to phenols has shed considerable insight on their role in drug metabolism and cytotoxicity. Carbonium ions were shown to be intermediates in these reactions. The results allow firm predictions to be made as to biological lifetime of an arene oxide, ratio of product phenols which can be expected, and reactive intermediates such as new arene oxides and ketones which might be formed. Thus, a physical-chemical basis now exists for a priori prediction of the phenols, dihydrodiols, and glutathione conjugates that will form from a particular arene oxide. Interestingly, only the relatively stable arene oxides have been implicated in liver necrosis and carcinogenesis.

In vivo studies of the metabolism of simple substituted benzenes have shown that compounds which have electron withdrawing groups show a primary isotope effect on hydroxylation at the position *meta* to the substituent. This observation is inconsistent with an arene oxide intermediate and suggests a direct insertion mechanism catalyzed by a different hepatic enzyme.

Investigation of the hydroxylation of chlorobenzene with liver microsomes and with the soluble preparations of hepatic cytochrome P450 and P448, revealed a marked variation in the ratio of isomeric phenols. The *ortho-* and *para*-chlorophenols are formed, respectively, from isomerization of the 2,3- and 3,4-arene oxidex, while the *meta*-chlorophenol appears to be formed by a direct hydroxylation pathway.

Examination of the substrate specificity of glutathione-S-epoxide transferase have shown wide differences in reactivity of arene oxides - those of polycyclic hydrocarbons being most reactive. These observations point to the importance of such enzymes as a protection mechanism aganist necrosis and carcinogenesis.

Solubilized epoxide hydrase has now been separated from catalytically active cytochrome P-450, and preliminary observations suggest that several hydrases are actually present based on differential purification factors. Studies on the mechanism of action of the enzyme in liver microsomes indicate that sterric rather than electronic factors are most important.

Work in the area of physical organic chemistry has led to the first preparation of a *cis* aldimine and has established both the sign and magnitude of H-C=^{15}N-coupling constants in these systems. This information is of value in structure determination and important to the general theory of spin-spin coupling. γ-Hydrogen rearrangement has been established as the principal mode of decomposition of the C_7H_8 + ion in the mass spectrometer by investigation of deuterium-labeled compounds.

Significance to Biomedical Research and the Program of the Institute: Enzymatic oxidation reactions are extremely important in many biosynthetic pathways and in the metabolism, detoxication and action of most drugs.

Proposed Course of Project: The mechanism(s) of enzymatic hydroxylation and epoxidation will be further investigated. Arene oxides of other aromatic substrates will be synthesized and attempts will be made, using tracer techniques, to demonstrate their intermediacy in aryl oxidation. The mechanism of action of epoxide hydrase and glutathione transferase and their role in drug detoxification will be further studied. Assays for the hydration of polycyclic arene oxides will be developed and the relevant enzymes will be investigated. Attempts to relate the effect of labile arene oxides on cells grown in culture and in whole animals to the cytotoxicity and carcinogenicity of the parent hydrocarbons will be made. The effect of various parameters on the stereochemical opening of epoxides with the microsomal epoxide hydrases will be explored. Studies on model systems including photolysis of N-oxides will be continued.

Honors and Awards: None

Publications:

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Serial No. NIAMDD-LC-49
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Pharmacodynamic Amines and Enzymes Involved in Their Metabolism.

Previous Serial Number: NIAMDD-LC-54

Principal Investigator: Cyrus R. Creveling

Other Investigators: J. Daly and J. Lundstrom

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W. Lutz, Manchester College, North Manchester, Indiana
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B. Highman, NIAMDD-LEP
M. Jeffrey, NIAMDD-LBC

Man Year

Total: 4.6
Professional: 2.3
Other: 2.3

Project Description:

Objectives: To study the basic chemistry and biochemistry of the catecholamines, phenolic amines, indolealkylamines, imidazolealkylamines and their precursors and transformation products. To develop new techniques for the investigation of transport metabolism and binding phenomena as related to these amines and their interactions with various drugs. To isolate and purify enzymes operative in the biosynthesis and metabolism of biogenic amines. To determine the physical properties of these enzymes and the effect of pH, temperature, substrate specificity, reversible and irreversible inhibitors and the ionic composition of the incubation media. To develop assays and to apply these enzymatic assays to determine specific biogenic amines and to study the distribution and variation of enzyme levels. To develop new pharmacologically active agents for the study of the biological parameters that affect biogenic amines.

Methods Employed: Drugs and substrates labeled with tritium, deuterium or ^{14}C have been used to study the metabolic fate of endogenous amines and amino acids and the underlying mechanisms of uptake, release and metabolism of these compounds. Standard methods of enzyme purification have been used including: differential centrifugation, density gradient centrifugation, gel filtration, electrophoresis, standard column chromatography, immunodiffusion, immunoelectrophoresis and affinity labeled column chromatography.

Major Findings: Purification of catechol-O-methyltransferase from the soluble fraction of rat liver has now afforded a single protein species with a molecular weight of 23700. Amino acid analysis indicates the presence of two cysteine moieties in this form of catechol-O-methyltransferase. Inactivation studies with ^{14}C-N-ethylmaleimide and other sulfhydryl reagents indicate that one sulfhydryl group is present in the active center of the enzyme and is related to the binding of S-adenosylmethionine. Another form of catechol-O-methyltransferase with an apparent molecular weight from 37000-46000 was also isolated. Antibodies have been prepared by administration of the low molecular weight form to rabbits. Initially, antibodies reacting as a single species with only the higher molecular weight form were formed. A second antibody appeared 20 to 30 days later in all animals which interacted with the low molecular form. Both antibody preparations cross-react with crude catechol-O-methyltransferase from liver, heart, kidney, of various rat strains, including the spontaneously hypertensive Aoki-wistar rat, various mouse strains, and guinea pig. Cross-reaction was not obtained with monkey, cat and dog. The antibodies cross-react with enzyme in crude liver microsomes prepared from rat, mouse and guinea pig and with the same enzyme in a solubilized preparation from rat liver.

Long term destruction of the cardiac uptake mechanism and storage sites for norepinephrine, elicited by certain tri- and tetrahydroxyphenethylamines, is enhanced by monoamine oxidase inhibitors. In contrast, long term destruction elicited by 5,7- and 6,7-dihydroxytryptamines is prevented by inhibition of monoamine oxidase. 5,6-Dihydroxytryptamine does not cause destruction of the uptake mechanism and storage sites for norepinephrine, but both it and to a greater extent, 6,7-dihydroxytryptamine, cause necrotic lesions in both heart and blood vessels. These effects appear to be blocked by inhibition of monoamine oxidase. The results indicate that destruction of cardiac noradrenergic terminals is dependent upon both a high affinity for neuronal uptake and a rapid rate of autoxidation in the phenethylamine series, while in the dihydroxytryptamine series a cytotoxic metabolite would appear to be formed by the action of monoamine oxidase. All of the dihydroxytryptamines autoxidize at a slow rate compared to that of 6-hydroxydopamine. Structural requirements for inhibition of cardiac uptake of radioactive norepinephrine have now been defined for both phenethylamines and tryptamines.

5,7-Dihydroxytryptamine has been found to be more active than 5,6-dihydroxytryptamine with respect to selective destruction of serotonin-containing neurons in the central nervous system.

A colchicine binding protein has been isolated from bovine brain and its behavior on acrylamide disc gel electrophoresis examined. Two monomeric forms with molecular weights of 50000 and 56000 have been obtained. Sulfhydryl determinations with ^{14}C-N-ethylmaleimide have shown 28 to 30 sulfhydryl groups per mole of both monomeric forms.

Significance to Biomedical Research and the Program of the Institute: The biosynthesis and metabolism of biogenic amines are of fundamental importance to the fields of neurochemistry, pharmacology and have a direct bearing on the understanding of the functioning of the cardiovascular system.

Proposed Course of Project: The binding and release of norepinephrine as influenced by drugs will be explored further, as will interactions of certain amines and drugs at storage and receptor sites. Studies on active site labeling and the primary structure of catechol-O-methyltransferase will be continued. Studies on inhibitors of tyrosine hydroxylase, phenylalanine hydroxylase, dopamine-β-hydroxylase and catechol-O-methyltransferase will be continued. The possible significance of false transmitters for histamine and serotonin will be explored. An attempt to rationalize the kinetic interactions of COMT, substrate, metal ion and S-adenosylmethionine in terms of a reaction mechanism and the formation of an active methyl-enzyme intermediate is in progress. The interactions of thio analogs of phenolic and catecholic biogenic amines with a variety of enzymes and biological systems will be explored in greater detail.

Honors and Awards: None

Publications:

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ANNUAL REPORT
LABORATORY OF EXPERIMENTAL PATHOLOGY

Biology of Degenerative Joint Diseases (Dr. Sokoloff and staff)

As in previous years, the Section on Rheumatic Diseases has continued its investigations on the biology of degenerative joint disease. This major cause of rheumatic disability involves the interaction of aging and mechanical destruction of the movable joints.

The principal effort during the period has been directed at the behavior articular chondrocytes *in vitro* and the measurement of synthesis of specific cell products; and at an examination of articular tissues by light and electron microscopy.

The definitive chemical characterization of proteoglycans synthesized by rabbit articular chondrocytes under various conditions of culture has been completed. It discloses major differences between chondrocytes and dermal fibrocytes. Under spinner conditions, the products of chondrocytes resemble closely those of intact articular cartilage in organ culture despite the fact that previously in monolayer culture the cells were only protodifferentiated. Aside from a small fraction of hyaluronate, surprising findings included the presence of disulfated chondroitins and dermatan sulfate. This did not result from contamination by extraneous connective tissue because serial histological sections showed none; presumably it reflected a considerable heterogeneity of cartilage, a phenomenon increasingly being recognized by workers in this field.

Biological characteristics of a pituitary mitogen for articular chondrocytes described in the 1971 report have been further elaborated. It reduced the generation time from 16 hours to 10. This can be accounted for by a virtual obliteration of the G_1 phase of the cell cycle. A reduction of radiosulfate incorporation did not reflect a specific suppression of mucopolysaccharide synthesis or dedifferentiation to a fibroblast since hyaluronate, RNA and protein synthesis also were reduced. The effect was observed under spinner conditions as well but less so than in monolayer culture. It was reversible. The velocity of uptake of deoxyglucose was increased by the preparation.

Culture of cartilage cells from 16 human (age 13 to 58 years) amputation specimens yielded confluent primary monolayer growth in each instance. The doubling time, however, was very long (more than two weeks) and growth in subculture was difficult. Skin fibrocytes differed from articular chondrocytes morphologically, in ease of subculture and in their short doubling time. Cells from a case of osteoarthritis secondary to multiple epiphyseal dysplasia behaved like fibrocytes.

The anatomic findings in previous projects have been extended. Amianthoid (asbestoid) fibers in aging human costal cartilage were shown to be collagenous both by their electron microscopic appearance and solubilization by clostridial collagenase. The fibers differed morphologically from collagen of dermis, synovium, tendon, fibro- and hyaline articular cartilage in dimension, contour and arrangement. They measured up to 1 μ in width and the period length was 560 - 620 Å. Their thickness, straightness and parallel organization suggest that amianthoid fibers are rigid and conceivably have many intermolecular crosslinks.

124 of 148 "randomly sampled" femoral heads obtained from total hip replacement procedures have been examined systematically. These included 50 cases of primary and 18 of secondary osteoarthritis; and 19 cases of nontraumatic aseptic necrosis. The greatest preoperative diagnostic problem was aseptic necrosis. Errors occurred in both directions. The anatomic basis for the roentgenographic appearances of this lesion have been refined as a result. The Marchi technique (indicated in last year's report) did not eliminate the technical artefacts which confuse evaluation of fat embolism in the pathogenesis of the necrosis.

A gross, radiologic and histological examination of human spines has been carried out to examine the role of vertebral arteries in the pathogenesis of cervical spondylotic myelopathy. 60 post-mortem specimens are in various stages of technical preparation.

Amyloidosis (Dr. G. G. Glenner and staff)

An amyloid fibril protein of unknown origin: Following the first amino acid sequence analysis of an amyloid fibril protein and the discovery of its immunoglobulin origin, the major protein obtained from amyloid fibril preparations derived predominantly from patients with the "secondary" form of the disease was subjected to this method of analysis. The result of these amino acid sequence analyses revealed the existence of a further class of amyloid fibril proteins. Unlike the major protein obtained from amyloid fibrils of immunoglobulin origin, however, the major amyloid fibril protein from this group of cases had an almost identical sequence i.e. Arg-Ser-Phe-Phe-Ser-. Differences based on the presence or absence of arginine as the amino-terminal amino acid and differences in the molecular weights and/or the amino acid composition of the individual proteins reported indicated that, as with many amyloid fibril proteins of immunoglobulin origin, these proteins may have been derived from a large protein by proteolytic digestion. The nature of the protein of origin of this class of amyloid fibril proteins is, as of this writing, not known. By immunochemical methods we have demonstrated the presence of an immunoglobulin protein constituting a lesser proportion of these amyloid fibril preparations; this would imply that amyloid fibril formation in these cases also is linked to the deposition of the whole or a portion of an immunoglobulin molecule. It is, however, not known as yet whether the immunoglobulin protein component is homogeneous as in the case of amyloid fibrils of immunoglobulin origin.

Although the majority of the cases shown to have this class of amyloid protein have been obtained from patients with associated inflammatory diseases (e.g., rheumatoid arthritis), one of the patients was not known to have an associated inflammatory disorder. Conversely, it has been demonstrated that the major fibril protein obtained from one patient having amyloidosis associated with two distinct inflammatory diseases was of immunoglobulin origin. So far the presence of the amyloid protein of unknown origin (AUO) has not been demonstrated chemically in cases in which the major fibril protein is of immunoglobulin origin. It is possible that not until an antiserum is obtained to the AUO protein can a judgement be made as to whether it is a constituent of all amyloid fibril preparations. Therefore, although it is tempting to speculate that all amyloid fibrils are constituted in differing proportions of both immunoglobulin and "nonimmunoglobulin" protein, for the present it would seem advisable not to assume that all cases even of inflammation-associated amyloidosis will invariably be associated with this AUO protein as either a major or minor component of the fibril. The similarity, however, in both the clinical and pathologic manifestations of "primary" and "secondary" amyloidosis suggests that a similar, but not necessarily identical, pathologic process occurs in both.
Synthetic amyloid fibrils: It has been shown that polymeric aggregates of the variable region of some Bence Jones as well as poly-*l*-lysine in its β-conformation have the polarization properties of Congo red-stained amyloid fibrils. Insulin and glucagon, proteins with strikingly different primary structures, can be polymerized to form parallel and anti-parallel β-pleated sheet fibrils, respectively, as defined by infrared spectroscopic and x-ray crystallographic analysis. Under appropriate conditions of polymerization the fibrils formed are indistinguishable from those seen in many cases of amyloidosis. These protein polymers have polarization optical properties of Congo red-stained amyloid fibrils over a wide range of induced variation in their ultrastructural morphology. Prostatic corpora amylacea, which have similar properties but are composed of concentrically arranged fibrils, have recently been demonstrated to have x-ray diffraction features consistent with a β-structure. The presence of the β-pleated sheet as the major conformation, therefore, may be the underlying feature that distinguishes proteins having the properties of Congo red-stained amyloid fibrils from those, e.g. fibrin, that do not. We conclude that the terms "amyloid" and "amyloidosis" are generic terms. That is, fibrillar deposits of proteins having a β-conformation, regardless of chemical composition will be classified as "amyloid" when Congo red birefrigence is used as a defining property. The "amyloid" associated with insulinomas and the pancreatic islets in diabetes may be derived, for example from insulin.

Cytogenetic Studies (Dr. J. H. Tjio, Dr. B. White and staff)

Pattern of recovery of hemopoiesis in the mouse after irradiation and mechanical depopulation: The TiAld mouse strain represents a Robertsonian fusion of two chromosome pairs and is derived from the AKR parent strain. It lends itself ideally to the production of radiation-induced chimaeras in which 97-100% of the bone marrow is of T1Ald karyotype, while the bone itself is of the AKR type. This system was used in cooperation with Dr. W. W. Smith (NCI-LPHY) and Dr. George Brecher (University of California Medical Center, San Francisco) to investigate the possible role of a common precursor cell of both bone and marrow following repopulation of a mechanically depopulated mouse femur. After lethal irradiation of the AKR host, T1Ald marrow was transplanted; subsequently, one femur of the recipient was mechanically depopulated. While "local" regeneration occurred, the marrow cells were of donor (T1Ald) type rather than host (AKR) type. Thus in this system the derivation of bone marrow from the postulated common precursor cell of bone and marrow can be excluded, since such a cell would be of host origin.

In other experiments with the same system, the previous experience with T6T6 markers was confirmed, that marrow transplants do not "take" in normal unirradiated recipients, even though the genetic constitution of the AKR and T1Ald mice are presumably identical. Experiments are now planned to explore various possibilities to explain this phenomenon. Possibly, loss of chromosome material during fusion accounts for a minute difference and consequent immunologic incompatibility, which is nullified by irradiation. Another, more intriguing, possibility is that marrow stem cells are not in cycle and, although they may be seeded in normal recipients, proliferate only in irradiated recipients in which various hemopoietins or other factors stimulate the stemcell proliferation. These alternatives are now under investiagion.

Clinical and cytogenetic studies on human congenital and developmental disorders: The differential staining methods of quinacrine mustard fluorescence and Giemsa-banding are being applied in our laboratory to studies of patients with various genetic disorders, abnormalities of gonadal development and function, and selected cases of infertility in the male. Since in many such patients no gross chromosomal abnormalities are detectable using routine cytogenetic methods, the differential staining procedures provide a means to detect small structural changes in chromosomes. A rearrangement that does not produce an obvious change in chromosome morphology can often be identified by altered banding patterns in the chromosomes involved, such as the duplication or deletion of chromosomal segments, peri-centric inversion, and some cases of reciprocal translocation. Since the Y chromosome has a characteristic intensely fluorescent region and the X chromosome has a characteristic Giemsa-banding pattern, differential staining analysis of these chromosomes enables us to make a more precise diagnosis in many patients with abnormal gonadal development and function.

A preliminary quinacrine-mustard staining study of sperm from infertile males with oligospermia or abnormal sperm morphology has been completed (in cooperation with Dr. R. Sherins, RR-NICHD). Of 18 patients sampled, two showed frequent double Y-chromatin bodies (9 and 18%); these samples had very abnormal sperm morphology. Since double Y-bodies can reflect nondisjunction of the Y at second meiotic metaphase in those with a normal XY karyotype or nondisjunction at first meiotic metaphase in those with and XYY karyotype, peripheral blood karyotypes will be necessary to follow up these two samples. Fourteen samples showed no double Y-bodies in the sperm, and two samples had one double Y-body (1%). These findings indicate that, except for very abnormal smears, the incidence of double Y-bodies is very low or zero in this group of patients. This is an even lower figure than that reported by others for normals (2%), and is more consistent with the low incidence of XYY males observed in the newborn male population.

Histochemical and Ultrastructural Studies: Methods and Applications (Dr. Feder and staff)

Beta-glucuronidase as a cell marker: In tetraparental mice derived by fusing high-glucuronidase (C57BL) and low-glucuronidase (C3H) strains, the mosaicism of the liver can be made apparent by means of the histochemical stain for beta-glucuronidase (Mintz, Proc. Nat. Acad. Sci. 69: 2032, 1971). Fixation techniques are being worked out in this laboratory to make this approach applicable to other cell types. In this way it may be possible to determine cell origins in transplanted tissues and to reconstruct cell migrations during embryonic development (with Dr. Mullen and Dr. Sidman).

Marking neurons for light and electron microscopy: A promising approach to understanding neural integration is the microscopic identification and examination of individual neurons after the neuron has been injected with a marker dye through an intracellular microelectrode. The application of this method is limited at present by the very weak fluorescence of the dye that is used (Procion Yellow). Work is in progress to synthesize a dye which, in addition to intense fluorescence, must have an over-all negative charge, high solubility in water and the ability to bind covalently to protein (with Mr. Stewart).

Studies on developing nerve tissue: Several studies, primarily with the light and electron microscope, are in progress on genetic lesions affecting cerebellar development and function. In the mutant mouse "staggerer", there is a congenital ataxia apparent soon after birth. During the early development of the cerebellum, the granule and Purkinje cell populations fail to undergo their normal interactions, resulting in granule cell hypoplasia, absence of synapses between parallel fibers and Purkinje cells, and finally degeneration of virtually all granule cells. The primary defect appears to involve

Purkinje cells. In a human disorder in which the predominant effect is on Purkinje cells (olivopontocerebellar degeneration, and autosomal dominant trait), two cerebellar biopsies have been examined. Within the Purkinje cells, two striking and characteristic types of inclusions have been seen, one of which resembles inclusions associated with viral diseases. In other studies we are using a new approach to cell-cell interactions in developing nerve tissue. Freeze-fracture techniques have revealed new specializations of the surface membranes at which neurons and glia are in contact with themselves and each other (with Dr. D. Landis, Dr. S. Landis and Dr. Reese).

Thyroid peroxidase cytochemistry: The effects of TSH on the distribution of thyroperoxidase were investigated using cytochemical methods. Although the enzyme is normally present at the apical cell surface of the thyroid follicular cell it is absent from the distal surfaces of pseudopods and is not present on the membranes of colloid droplets. These findings suggest that either the membrane of pseudopods is synthesized via different mechanisms than is normal apical plasma membrane, or that it is modified during the course of pseudopod formation with the result that peroxidase activity is lost (Drs. Tice and Wollman). In additional studies, a microtome seismometer, the "chatterbox" was developed to detect at the ultramicrotome vibrations which cause chatter in ultrathin sections. Using a modified phonograph pickup mounted on the specimen and a suitable amplifying chain, it was found that these vibrations could be easily heard and distinguished from the other sounds accompanying sectioning. This relatively simple divice should result in large time economies for microtomists, for it permits this common class of section defects to be detected without recourse to time-consuming electron microscopic examination of the sections (Drs Tice and McCutchen).

Structure and Biochemical Properties of Cell Membranes

Lymphocyte membrane glycoproteins: (Dr. B. T. Hourani with Dr. Pincus) Lymphocyte membrane proteins have been of primary interest over the past year. Previous work in other laboratories have been hampered by lack of sufficient amounts of lymphocytes and the long laborious fractionation schemes usually employed to yield purified plasma membranes. A rapid, reproducible method of membrane isolation using L 1210, a DBA/2 murine lymphoma grown in suspension cultures and available in large quantities was developed. Using polyethylene glycol-dextran two phase systems in the presence of $MgCl_2$ purified plasma membranes were obtained in 2-1/2 hrs total preparation time. The yield of total protein is comparable to the highest yields reported using laborious density gradient schemes.

The membranes, when extracted for glycoprotein by a modification of the Lithiumdiiodosalicylate method of Marchesi yield four unique glycoproteins with carbohydrate composition characteristic of membrane glycoprotein. When injected into rabbits antisera was reproducibly cytotoxic to L 1210 cells whereas preimmune sera was noncytotoxic. The glycoprotein extract inhibited agglutination of L 1210 cells by Lens culinaris hemagglutinin, wheat germ agglutinin and Concanavalin A thus indicating that carbohydrate determinants remained intact during the purification scheme. Milligram quantities of this glycoprotein extract have now been collected and fractionation of molecular species are underway. The specificity of the antisera is as well being evaluated.

Lectin purification and characterization of chemical and biologic preparation: Interest in lectins and their interaction with cell surfaces is currently investigated in many laboratories. Little, however, is known of the correlation between molecular species and biologic activity. Castor beans (Ricinis communis) contain protein(s) which react with and are cytotoxic for tumor cells but are also capable of agglutinating RBC's.

Two distinct agglutinins (Ricin I and Ricin II) were purified from castor beans by affinity chromatography, and each found to have cytotoxic and hemagglutinating activity. Ricin I is a tetrameric unit composed of two different subunits (2α and 2γ) which possesses potent hemagglutinating activity but relatively low cytotoxic activity. Ricin II is a dimer form composed of α and β subunits which has weak hemagglutinating activity but is highly cytotoxic. Molecular weights of the three subunits are in the 30,000 range. Ricin II has the molecular weight, amino acid composition and N-Terminal amino acid residues (Ala, ileu) characteristic of the Ricin D reported by others. These results suggest that each isolated component has both hemagglutinating and cytotoxic activity, a finding which differs from

Muscle cell surface mapping of the acetylcholine receptor: A third area of interest involved mapping of the acetylcholine receptors of chick myoblast in pure monolayer culture. Previous work in Dr. Gerald Fischbach's laboratory has shown that chicken embryo myoblasts have cell surface receptors for acetylcholine, and that α-Bungarotoxin, a protein isolated from the venom of Bungarus multicinctus is capable of specifically and irreversibly binding to receptors which block the binding of acetylcholine. The distribution of the receptors has been shown to be non-uniform over the cell surface using I^{125}-Bungarotoxin and radioautography. The attempt was made to map the receptor using Bungarotoxin-Ferritin conjugates and visualization of muscle cell surfaces using the freeze fracturing technique. Using glutaraldehyde to couple Bungarotoxin to Ferritin followed by gel filtration in high salt it was possible to purify the covalently bound Ferritin-Bungarotoxin complex and have data that the Ferritin is indeed distributed in a patchy nonrandom fashion over the muscle cell surface. Binding of Ferritin-Bungarotoxin is blocked by preincubation of myoblasts with curare and acetylcholine indicating specificity of binding. Complete mapping studies are underway as well as correlative electrophysiologic experiments with the Ferritin-Bungarotoxin complex (with Dr. Fischbach).

Immunochemical studies with histamine: (Dr. Ram)

It is not well established how histamine is held in the cell (in combination with heparin or a protein or by other means) or how it is released. It is therefore of some interest to see if antibodies specific for histamine can be experimentally obtained and whether such antibodies can be employed to throw light on the mechanisms of the manifold biologic functions of histamine.

Accordingly, conjugates of histamine with a number of proteins (rabbit gamma globulin-RGG; rabbit serum albumin-RSA; bovine serum albumin-BSA; egg albumin-EA; and lysozyme-Ly.) were prepared as follows. Histamine dihydrochloride (700 mg), 100 mg protein and 2.0 gm of ethyl carbodiimide were dissolved in 10 ml of water and allowed to react for 2 hrs at room temperature, exhaustively dialyzed against water and lyophilized. This procedure yields a conjugate containing 45-60 moles of histamine per mole of protein.

Rabbits were immunized with one of the conjugates, namely RGG-Histamine by a variety of routes and procedures. After five biweekly injections, the sera of immunized animals reacted weakly in gel diffusion reactions as follows: A single precipitin band was obtained when these early antisera were reacted with histamine conjugates of RGG, RSA, BSA and a control RGG preparation treated with the carbodiimide reagent alone. With the histamine conjugate of EA, two precipitin bands were obtained one of which merged with the precipitin band obtained with the above derivatives. In inhibition experiments, the above precipitin reactions were inhibited completely by histamine and L-histidine methyl ester, but not by L-histidine or an unrelated basic compound lysine. Further studies are continuing to extend and intrepret these findings. Histamine conjugated sepharose has been prepared for use in affinity chromatographic identification of the specificity of the antibody.

Cycasin research: (Dr. Laqueur)

The microscopic examination of several hundreds of rats exposed in utero to the alkylating compound methylazoxymethanol (MAM) has been essentially completed and submitted in part for publication in a monograph devoted to the problem of multiple primary malignancies. The compound MAM is the aglycone of cycasin, a naturally occurring carcinogen which has been studied by us over the past years. Transplacental tumor induction has now been successful in two strains of rats, namely the Sprague-Dawley strain and the inbred Fischer strain. The findings have now been compared with those neoplasms occurring in control rats of both strains as background or spontaneous tumors.

Whereas the spontaneously occurring neoplasms predominantly develop in the organs of the endocrine system (pituitary, adrenal, thyroid and gonads) and their incidence distinctly increases with age, the MAM induced tumors involve organs such as liver, kidney, the large and small intestine, brain and lung. The induced tumors develop earlier than the spontaneous neoplasms and their occurrence is influenced by the total dose, age and sex of the recipients.

Perhaps the two most interesting observations which stand out among several other findings were the following: The perinatal induction of neoplasms in the lungs is limited to a brief period extending from the last day of intrauterine life to the first day of life, a period lasting about 48 hours. Moreover, such pulmonary tumors develop not only at multiple sites in the lungs but usually are also found in litter mates. This suggests an unusual degree of sensitivity of the lungs to the carcinogen during a brief perinatal period. The second observation concerns the development of cerebral neoplasms. Although induction of gliomas (oligodendrogliomas) is possible with MAM over a broader period of time, a concentration of brain tumor in animals exposed during the perinatal period is noted. Comparing the incidence of brain tumors produced with methylazoxymethanol with those in our animals treated identically with ethynitrosourea (ENU), though incidence of brain tumors in rats exposed transplacentally to ENU was signficantly greater and nearly 100%. This suggests the possibility that ethylation is more important than methylation with respect to induction of cerebral neoplasms, a notion, we hope to be able to test in the future.

Serial No. NIAMDD-LEP-1
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Metabolic and carcinogenic effects of Cycas Circinalis, its glucoside cycasin and its aglycone, methylazoxymethanol, in conventional and germfree rats.

Previous Serial Number: Same

Principal Investigator: Gert L. Laqueur

Other Investigators: Dr. M. Spatz, Dr. H. Matsumoto, and Mr. E. G. McDaniel

Cooperating Units: LNNS-NINDS (Dr. Spatz)
LNE-NIAMDD (Mr. McDaniel)
University of Hawaii (Dr. Matsumoto)

Man Years:

Total: 4
Professional: 1
Other: 3

Project Description:

As indicated in last year's project description a study concerned with transplacental carcinogenesis of methylazoxymethanol (MAM) the proximate carcinogen and mutagenically active compound of cycasin had been initiated in 1971. The detailed histologic examination of several hundreds of animals was completed and analyzed during the year covered by this report and prepared for presentation and publication at this years International Conference on Multiple Primary Cancers.

Because the induction period for tumors with MAM is long and not significantly shortened by transplacental exposure, it became necessary to support the analysis of induced tumors with an analysis of the tumors developing spontaneously in the strains of rats used by us and under our laboratory conditions.

Whereas the spontaneously occurring neoplasms predominantly develop in the organs of the endocrine system (Pituitary, adrenal, thyroid and gonads) and their incidence distinctly increases with age, the MAM induced tumors involve organs such as liver, kidney, the large and small intestine, brain and lung. The induced tumors develop earlier than the spontaneous neoplasms and their occurrence is influenced by the total dose, age and sex of the recipients.

Perhaps the two most interesting observations which stand out among several other findings were the following: The perinatal induction of neoplasms in the lungs is limited to a brief period extending from the last day of intrauterine life to the first day of life, a period lasting about 48 hours. Moreover, such pulmonary tumors develop not only at multiple sites in the lungs but usually are also found in litter mates. This suggests an unusual degree of sensitivity of the lungs to the carcinogen during a brief perinatal period. The second observation concerns the development of cerebral neoplasms. Although induction of gliomas (oligodendrogliomas) is possible with MAM over a broader period of time, a concentration of brain tumor in animals exposed during the perinatal period is noted. Comparing the incidence of brain tumors produced with methylazoxymethanol with those in our animals treated identically with ethynitrosourea (ENU), the incidence of brain tumors in rats exposed transplacentally to ENU was significantly greater and nearly 100%. This suggests the possibility that ethylation is more important than methylation with respect to induction of cerebral neoplasms, a notion, we hope to be able to test in the future.

Publications:

Laqueur, G. L.: Cycasin and Related Compounds in Proceedings 10th International Cancer Congress, Houston, Clark, R. L., Cumley, R. W. McCay, J. E. and Copeland, M. D. (eds): Oncology. 5, 196-202, 1970.

Laqueur, G. L., and Spatz, M.: Transplacental Induction of Tumours and Malforamtions in Rats with Cycasin and Methylazoxymethanol in WHO, IARC Scientific Publications No. 4 Transplacental Carcinogenesis pp 59-64, 1973.

Serial No. NIAMDD-LEP-2
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Preparation of stained tissue sections for investigation and diagnostic purposes.

Previous Serial Number: NIAMD-LEP-3

Principal Investigator: Miss Virginia Reveal - Head, Tissue Preparation Laboratory

Other Investigators: None

Man Years:

Total: 3
Professional: 0
Other: 3

Project Description:

The statistical report of this unit is shown below.

	Specimen	Stained Slides		Spare	
	Accessioned	Routine	Special	Slides	Total
Animals	1557	4046			
Surgical	694	1754			
		5800	6261		
		12061		10057	22118

Serial No. NIAMDD-LEP-3
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Protein-protein interactions and immune processes in normal and disease states.

Previous Serial Number: None

Principal Investigator: J. Sri Ram, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Projects covered during this report period included continuation of some of the earlier studies interrupted due to assignment abroad and setting up of an Immunochemistry Laboratory in the new quarters made available. These studies, related to the antigenicity of glutathione, histamine and creatine phosphokinase, were necessarily limited to a narrow immunochemical area without extension into histochemical and immunopathologic areas as previously envisaged, due to the as yet incomplete nature of the present laboratory set-up.

Glutathione as Hapten: Attempts to obtain antibodies specific for this tripeptide were continued employing the technique of using a glutathione-carrier protein complex as the immunizing vehicle. Strong suggestive evidence has recently been obtained for the presence of glutathione specific antibodies in the antisera. In these studies immunization of rabbits was carried out with protein conjugates (rabbit and bovine albumin) obtained with carbodiimide as well as glutaraldehyde as conjugating agents. Tests for the specificity of the antisera were carried out with glutathione conjugated to unrelated carriers such as lysozyme, polyglutamic acid and egg albumin, these derivatives being prepared under identical conditions as the immunizing conjugates. In one study a protein derivative with S^{35}-glutathione was also prepared and characterized. Gel diffusion and complement fixation tests have given evidence for the presence of a weak antibody specific for glutathione moiety. Attempts are now underway to improve the antibody titer by prolonged immunizations as well as changing the route and dosage of immunization etc. When antisera of suitable titers are available specific antibody globulin reacting with glutathione will be prepared by affinity chromatography. For this purpose glutathione conjuated sepharose has been prepared employing the cyanogen bromide technique.

Immunochemical studies with histamine: Among the pharmacologically active chemical agents, histamine is one of the most interesting. It is widely distributed in mammalian tissues particularly in mast cells, platelets and basophylic leucocytes of blood. Certain antigen-antibody reactions and drugs mediate the release of histamine in a soluble form which then causes a number of pharmacologic and inflammatory reactions such as anaphylaxis, contraction of smooth muscles, vasodilation, increase in capillary permeability etc. It is not well established how histamine is held in the cell (in combination with heparin or a protein or by other means) or how it is released. It is therefore of some interest to see if antibodies specific for histamine can be experimentally obtained and whether such antibodies can be employed to throw light on the mechanisms of the manifold biologic functions of histamine.

Accordingly, conjugates of histamine with a number of proteins (rabbit gamma globulin-RGG; rabbit serum albumin-RSA; bovine serum albumin-BSA egg albumin-EA; and lysozyme-Ly.) were prepared as follows. Histamine dihydrochloride (700 mg), 100 mg protein and 2.0 gm of ethyl carbodiimide were dissolved in 10 ml of water and allowed to react for 2 hrs at room temperature, exhaustively dialyzed against water and lyophilized. This procedure yields a conjugate containing 45-60 moles of histamine per mole of protein.

Rabbits were immunized with one of the conjugates, namely RGG-Histamine by a variety of routes and procedures. After five biweekly injections, the sera of immunized animals reacted weakly in gel diffusion reactions as follows: A single precipitin band was obtained when these early antisera were reacted with histamine conjugates of RGG, RSA, BSA and a control RGG preparation treated with the carbodiimide reagent alone. With the histamine conjugate of EA, two precipitin bands were obtained one of which merged with the precipitin band obtained with the above derivatives. In inhibition experiments, the above precipitin reactions were inhibited completely by histamine and L-histidine methyl ester, but not by L-histidine or an unrelated basic compound lysine. Further studies are continuing to extend and interpret these findings. Histamine conjugated sepharose has been prepared for use in affinity chromatographic identification of the specificity of the antibody.

blications:

Ram, J. S.: Selected laboratory procedures in immunochemistry. Issued by Fulbright Foundation, India. 1972.

Serial No. NIAMDD-LEP-4
1. Experimental Pathology
2. Chemical Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1973

Project Title:
1) Lymphocyte membrane glycoproteins.
2) Lectin purification and characterization of chemical and biologic properties.
3) Muscle cell surface mapping of the acetylcholine receptor.

Previous Serial Number: None

Principal Investigator: Benjamin T. Hourani

Other Investigators: Drs. Marchesi and Tomita

Cooperating Units: D-LM (Dr. J. Pincus)
CH-BB (Dr. G. Fischbach)

Man Years:

Total: 4
Professional: 1
Other: 3

Project Description:

Lymphocyte membrane glycoproteins: Lymphocyte membrane proteins have been of primary interest in our laboratory over the past year. Previous work in other laboratories has been hampered by lack of sufficient amounts of lymphocytes and the long laborious fractionation schemes usually employed to yield purified plasma membranes.

We initially spent several months in finding a rapid, reproducible method of membrane isolation using L 1210, a DBA/2 murine lymphoma grown in suspension cultures and available in large quantites. Using polyethylene glycol-dextran two phase systems in the presence of $MgCl_2$ we have obtained purified plasma membranes (with E.M. criteria, enzyme marker criteria) in 2-1/2 hrs total preparation time. The yield of total protein is comparable to the better yields reported using laborious density gradient schemes.

The membranes, when extracted for glycoprotein by a modification of the lithiumdiiodosalicylate method of Marchesi yield four unique glycoproteins with carbohydrate composition characteristic of membrane glycoprotein. When injected into rabbits, antisera is reproducibly cytotoxic to L 1210 cells and preimmune sera noncytotoxic. The glycoprotein extract inhibits agglutination of L 1210 cells by *Lens culinaris* hemagglutinin, wheat germ agglutinin and Concanavalin A thus indicating that carbohydrate determinants remain intact during the purification scheme. Milligram quantities of this glycoprotein extract have now been collected and fractionation of molecular species are underway. Specificity of antisera is as well being evaluated.

Lectin purification and characterization of chemical and biologic preparation: Interest in lectins and their interaction with cell surfaces is currently a hot bed of investigation in many laboratories. Little, however, is known of the correlation between molecular species and biologic activity. So we sought to investigate and characterize both chemically and biologically a lectin with two known properties.

Castor beans (Ricinis communis) contain protein(s) which react with and are cytotoxic for tumor cells but are also capable of agglutinating RBC's. Two distinct agglutinins (Ricin I and Ricin II) were purified from castor beans by affinity chromatography, and each found to have cytotoxic and hemagglutinating activity. Ricin I is a tetrameric unit composed of two different subunits (2 α and 2 γ) which possesses potent hemagglutinating activity but relatively low cytotoxic activity. Ricin II is a dimer form composed of α and β subunits which has weak hemagglutinating activity but is highly cytotoxic. Molecular weights of the three subunits are in the 30,000 range. Ricin II has the molecular weight, amino acid composition and N-Terminal amino acid residues (Ala, ileu) characteristic of the Ricin D reported by others. These results suggest that each isolated component has both hemagglutinating and cytotoxic activity, a finding which differs from previous reports.

Muscle cell surface mapping of the acetylcholine receptor: The third area of interest involves mapping of the acetylcholine receptor of chick myoblasts in pure monolayer culture. Previous work in Dr. Gerald Fischbach's laboratory has shown that chicken embryo myoblasts have cell surface receptors for acetylcholine and that α-Bungarotoxin, a protein isolated from the venom of Bungarus multicinctus is capable of specifically and irreversibly binding to receptors which block the binding of acetylcholine. The distribution of those receptors has been shown to be nonuniform over the cell surface using I^{125}-Bungarotoxin and radioautography. We therefore sought to map the receptor using Bungarotoxin-Ferritin conjugates and visualization of muscle cell surfaces using the freeze fracturing technique. Several laboratories have attempted mapping previously but were unsuccessful. Initial attempts in our lab as well failed and we now feel that this was due to electrostatic attraction between the basically charged Bungarotoxin and the negatively charged Ferritin resulting in dissociation of the complex when mapping was attempted. Using glutaraldehyde to couple Bungarotoxin to Ferritin followed by gel filtration in high salt we were able to purify the covalently bound Ferritin-Bungarotoxin complex and have preliminary data that the Ferritin is indeed distributed in a patchy nonrandom fashion over the muscle cell surface. Binding of Ferritin-Bungarotoxin is blocked by preincubation of myoblasts with curare and acetylcholine indicating specificity of binding. Complete mapping studies are underway as well as correlative electrophysiologic experiments with the Ferritin-Bungarotoxin complex.

Publications:

Tomita, M., Hourani, B. T., and Marchesi, V. T.: Structure, hemagglutination and cytotoxicity of purified agglutinin from *Ricinus communis* seeds. Biochimica et Biophysica Acta.

Serial No. NIAMDD-LEP-5
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Prlject Report
July 1, 1972 through June 30, 1973

Project Title: Histochemistry: principles, methods and applications

Previous Serial Number: NIAMD-LEP-6

Principal Investigator: Ned Feder

Other Investigators: Michael V. L. Bennett, Richard Mullen, Thomas S. Reese, Richard L. Sidman, Walter W. Stewart

Cooperating Units: LNS-NINDS (Reese)
Albert Einstein Medical School (Bennett)
Harvard Medical School (Mullen, Sidman)
Harvard University (Stewart)

Man Years:

Total: 2.2
Professional: 1
Other: 1.2

Project Description:

Beta-glucuronidase as a cell marker: In tetraparental mice derived by fusing high-glucuronidase (C57BL) and low-glucuronidase (C3H) strains, the mosaicism of the liver can be made apparent by means of the histochemical strain for beta-glucuronidase (Mintz, Proc. Nat. Acad. Sci. 69: 2032, 1971). Fixation techniques are being worked out in this laboratory to make this approach applicable to other cell types. In this way it may be possible to determine cell origins in transplanted tissues and to reconstruct cell migrations during embryonic development (with Dr. Mullen and Dr. Sidman).

Marking neurons for light and electron microscopy: A promising approach to understanding neural integration is the microscopic identification and examination of individual neurons after the neuron has been injected with a marker dye through an intracellular microelectrode. The application of this method is limited at present by the very weak fluorescence of the dye that is used (Procion Yellow). Work is in progress to synthesize a dye which, in addition to intense fluorescence, must have an over-all negative charge, high solubility in water and the ability to bind covalently to protein (with Mr. Stewart).

Critique of work on scotophobin: A detailed and critical analysis has been made of a report by Ungar, Desiderio and Parr on the chemistry of scotophobin. This analysis, mentioned in last year's project report, has now been published.

Serial No. NIAMDD-LEP-6
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Thyroid peroxidase cytochemistry

Previous Serial Number: NIAMD-LEP-7

Principal Investigator: Lois W. Tice

Other Investigators: Dr. Seymour Wollman
Dr. C. W. McCutchen

Cooperating Units: LP-NCI

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

The effects of TSH on the distribution of thyroperoxidase were investigated using cytochemical methods. Although the enzyme is normally present at the apical cell surface of the thyroid follicular cell, it is absent from the distal surfaces of pseudopods and is not present on the membranes of colloid droplets. These findings suggest that either the membrane of pseudopods is synthesized via different mechanisms than is normal apical plasma membrane, or that it is modified during the course of pseudopod formation with the result that peroxidase activity is lost.

Studies are in progress to determine the effects of hypophysectomy and subsequent TSH administration of the distribution of thyroperoxidase. Apical activity is lost within a few days after hypophysectomy (and apical vesicles within 5 - 7 days) but enzyme activity is retained in the nuclear envelope and endoplasmic reticulum for long periods (8 weeks) after operation, suggesting that the enzyme is not induced by TSH. if TSH is given to animals hypophysectomized for 3 weeks, substantial expansion of the Golgi apparatus is seen within 24 hours, and apical activity returns within 48 hours. Present results tentatively suggest that the control point for secretion of peroxidase into the follicular lumen lies at the level of the Golgi apparatus.

In additional studies, a microtome seismometer, the "chatterbox" was developed to detect at the ultramicrotome vibrations which cause chatter in ultrathin sections. Using a modified phonograph pickup mounted on the specimen and a suitable amplifying chain, we found that these vibrations could be easily heard and distinguished from the other sounds accompanying sections. This relatively simple device should result in large time economies for microtomists, for it permits this common class of section defects to be detected without recourse to time-consuming electron microscopic examination of the sections.

Publications:

None

Serial number NIAMDD-LEP-7
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Ultrastructural studies on cerebellar cortex.

Previous Serial Number: none

Principal Investigator: Dennis M. D. Landis

Other Investigators: Dr. Richard L. Sidman and S. C. Landis

Cooperating Units: Department of Neuropathology, Harvard Medical School

Man Years

Total: 1
Professional: 1
Other: 0

Project Description:

Ultrastructure of the cerebellar cortex in human autosomal dominant olivopontocerebellar degeneration: Two cerebellar biopsies obtained from affected patients by posterior fossa craniotomy were prepared for light and electron microscopic examination in an effort to establish the pathogenesis of neuronal death in olivopontocerebellar degeneration. Both biopsies revealed severe and progressing Purkinje cell degeneration, characterized by progressive pyknosis and peculiar membrane organizations. There is minor granule cell degeneration and filamentous degeneration of central axons in glomeruli. Two aspects of the Purkinje cell degenerative process have not been described elsewhere: a subsurface dendritic membrane specialization similar to postsynaptic membrane specializations but occurring in association with large vesicles and without a presynaptic element, and a crystalloid inclusion in Purkinje axon recurrent collaterals. The distribution of neuronal degeneration confirms previous light microscopic studies, and reveals unsuspected glomerular degeneration. Most of the morphologic changes during Purkinje cell degeneration are nonspecific, but there are present crystalloid inclusions comparable to those which appear in viral injections. This raises the possibility that infectious agents may participate in the disease process.

Cerebellar cortical development in the staggerer mouse: In order to investigate the effects of the sg gene on cerebellar cortical morphogenesis during postnatal development, C57BL/6J mice of genotype se d +/se d + were crossed to yield progeny of genotypes se d +/se d +, se d +/+ + sg, and + + sg/+ + sg, in the ration 1:2:1. Littermate + + sg/+ + sg and se d +/se d + mice were prepared for light and electron microscopic examination at 1,3,5,7,10,14, 19,21,33, and 43 days postnatal. The external granular layer of staggerer mice is hypoplastic throughout postnatal development, and disappears prematurely. Granule cell differentiation is normal, except that parallel fibers never establish synaptic contact with Purkinje cells, and virtually the entire granule cell population later degenerates. Purkinje cells are present in approximately normal numbers, but are small, do not organize into the usual lamina, acquire only a vestigial dendritic arbor, and never elaborate dendritic spines. The cerebellar malformation appears to be caused by a block in the normal developmental interactions of Purkinje and granule neurons, including the specific failure to form the parallel fiber: Purkinje spine snyapse (all other synaptic classes are present). This is the first described instance of a genetic lesion affecting the interactions of developing cell populations, and the first time it has been possible to relate a specific synaptic defect with an isolated abnormal gene.

Publications:

None

Serial No. NIAMDD-LEP-8
1. Experimental Pathology
2. Rheumatic Diseases
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Pathogenesis of experimental arthritis and pathology of rheumatism.

Previous Serial Number: NIAMD-LEP-8

Principal Investigator: Leon Sokoloff, M.D.

Other Investigators: Aubrey J. Hough, M.D.
Vishwa M. L. Srivastava, Ph.D.

Cooperating Units: NIAMDD:LBM (Jean W. Hickman, Ph.D.)
University of Vermont (John H. Bland, M.D.)

Man Years:

Total: 6
Professional: 3
Other: 3

Project Description:

Objectives:

1) Investigation of factors influencing development of degenerative joint disease in experimental animals and man.

2) Extending descriptive pathology of human rheumatic disease.

Methods Employed:

1) Articular chondrocytes are cultured *in vitro* and synthesis of specific cell products is measured.

2) Articular tissues are examined by light and electron microscopy.

3) Rheological properties of synovial fluid are studied with a specially designed apparatus.

Major Findings:

1) The definitive chemical characterization of proteoglycans synthesized by rabbit articular chondrocytes under various conditions of culture has been completed. It discloses major differences between chondrocytes and dermal fibrocytes. Under spinner conditions, the products of chondrocytes

resemble closely those of intact articular cartilage in organ culture despite the fact that previously in monolayer culture the cells were only protodifferentiated. Aside from a small fraction of hyaluronate, surprising findings included the presence of disulfated chondroitins and dermatan sulfate. This did not result from contamination by extraneous connective tissue because serial histological sections showed none; presumably it reflected a considerable heterogeneity of cartilage, a phenomenon increasingly being recognized by workers in this field.

2) Biological characteristics of a pituitary mitogen for articular chondrocytes described in the 1971 report have been further elaborated. It reduced the generation time from 16 hours to 10. This can be accounted for by a virtual obliteration of the G_1 phase of the cell cycle. A reduction of radiosulfate incorporation did not reflect a specific suppression of mucopolysaccharide synthesis or dedifferentiation to a fibroblast since hyaluronate, RNA and protein synthesis also were reduced. The effect was observed under spinner conditions as well but less so than in monolayer culture. It was reversible. The velocity of uptake of deoxyglucose was increased by the preparation.

3) Culture of cartilage cells from 16 human (age 13 to 58 years) amputation specimens yielded confluent primary monolayer growth in each instance. The doubling time, however, was very long (more than two weeks) and growth in subculture was difficult. Skin fibrocytes differed from articular chondrocytes morphologically, in ease of subculture and in their short doubling time. Cells from a case of osteoarthritis secondary to multiple epiphyseal dysplasia behaved like fibrocytes.

4) The anatomic findings in previous projects have been extended.

A. Amianthoid (asbestoid) fibers in aging human costal cartilage were shown to be collagenous both by their electron microscopic appearance and solubilization by clostridial collagenase. The fibers differed morphologically from collagen of dermis, synovium, tendon, fibro- and hyaline articular cartilage in dimension, contour and arrangement. They measured up to 1 μ in width and the period length was 560 - 620 Å. Their thickness, straightness and parallel organization suggest that amianthoid fibers are rigid and conceivably have many intermolecular crosslinks.

B. 124 of 148 "randomly sampled" femoral heads obtained from total hip replacement procedures have been examined systematically. These included 50 cases of primary and 18 of secondary osteoarthritis; and 19 cases of nontraumatic aseptic necrosis. The greatest pre-operative diagnostic problem was aseptic necrosis. Errors occurred in both directions. The anatomic basis for the roentgenographic appearances of this lesion have been refined as a result. The Marchi technique (indicated in last year's report) did not eliminate the technical artefacts which confuse evaluation of fat embolism in the pathogenesis of the necrosis.

C. A gross, radiologic and histological examination of human spines has been carried out to examine the role of vertebral arteries in the pathogenesis of cervical spondylotic myelopathy. 60 post-mortem specimens are in various stages of technical preparation.

D. Histologic studies performed between April 21, 1972 and April 9, 1973 include:

Joint	Other
89	64

Among the more illuminating cases were three of hyperostotic osteomalacia in familial hypophosphatemic rickets. As a result, biopsy procedures in future studies of this condition in the Clinical Center will be improved.

Proposed Course:

The project will terminate with the retirement of the principal investigator on July 1, 1973. Three papers describing the findings indicated above will be completed by that time. Several others will be completed thereafter. Dr. Hough will continue, during the following year, to collaborate with Dr. Sokoloff on several projects already under way (aseptic necrosis of human hips; histological heterogeneity of cartilage; fine structure of cement lines).

Publications:

Layman, D.L., Sokoloff, L, and Miller, E.J.: Collagen synthesis by articular chondrocytes in monolayer culture. Exper. Cell Res. 73: 107-112, 1972.

Malemud, C.J., and Sokoloff, L.: Rumalon and serum-free culture of chondrocytes. Arthritis Rheum. 15: 557, 1972.

Marcus, R.E.: The effect of low oxygen concentration on growth, glycolysis and sulfate incorporation by articular chondrocytes in monolayer culture. Arthritis Rheum. In press.

Marcus, R.E., and Srivastava, V.M.L.: Effect of low oxygen tensions on glucose-metabolizing enzymes in cultured articular chondrocytes.

Proc. Soc. Exptl. Biol. Med. In press.

Sokoloff, L.: The pathology of rheumatoid arthritis and allied disorders. In Hollander, J.L. and McCarty, D.J. (Eds.): *Arthritis and Allied Conditions* (ed. 8). Philadelphia, Lea and Febiger, 1972, pp. 309-332.

Sokoloff, L.: The pathology and pathogenesis of osteoarthritis. In Hollander, J.L. and McCarty, D.J. (Eds.): *Arthritis and Allied Conditions* (ed. 8). Philadelphia, Lea and Febiger, 1972, pp. 1009-1031.

Sokoloff, L.: A note on the histology of cement lines. In Kenedi, R. (Ed.): *Perspectives in Biomedical Engineering*. MacMillans. In press.

Sokoloff, L.: Mycoplasma (pleuropneumonia-like organism) arthritis. *Compar. Path. Bull.* In press.

Sokoloff, L., Malemud, C.J., Srivastava, V.M.L., and Morgan, W.D.: *In vitro* culture of articular chondrocytes. *Fed. Proc.* 32: 1499-1502, 1973.

Serial No. NIAMDD-LEP-9
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Protein and Proteolytic Enzyme Interrelationships in Normal and Disease States

Previous Serial Number: NIAMDD-LEP-9

Principal Investigator: George G. Glenner, M.D.

Other Investigators: S. Kimura, M.D., Visiting Associate (July 1, 1972-April 14, 1973), R. P. Linke, M.D., Visiting Associate (July 31, 1972-July 1, 1973), J. Kwiatkowska, Ph.D., Visiting Associate (March 6, 1973-July 1, 1973)

Cooperating Units: H. Bladen, Ph.D. (NIDR-LHP)
D. Eanes, Ph.D. (NIDR-LHP)
W. Terry, M.D. (NCI-I)
J. Termine, Ph.D. (NIDR-LHP)

Man Years:

Total:	5
Professional:	3
Other:	2

Project Description:

Amyloidosis

An amyloid fibril protein of unknown origin: Following the first amino acid sequence analysis of an amyloid fibril protein and the discovery in our laboratory of its immunoglobulin origin, the major protein obtained from amyloid fibril preparations derived predominantly from patients with the "secondary" form of the disease was subjected to this method of analysis. The result of these amino acid sequence analyses revealed the existence of a further class of amyloid fibril proteins. Unlike the major protein obtained from amyloid fibrils of immunoglobulin origin, however, the major amyloid fibril protein from this group of cases had an almost identical sequence, i.e. Arg-Ser-Phe-Phe-Ser-. Differences based on the presence or absence of arginine as the amino-terminal amino acid and differences in the molecular weights and/or the amino acid composition of the individual proteins reported indicated that, as with many amyloid fibril proteins of immunoglobulin origin, these proteins may have been derived from a larger protein by proteolytic digestion. The nature of the protein of origin of this class of amyloid fibril proteins is, as of this writing, not known.

By immunochemical methods we have demonstrated the presence of an immunoglobulin protein constituting a lesser proportion of these amyloid fibril preparations; this would imply that amyloid fibril formation in these cases also is linked to the deposition of the whole or a portion of an immunoglobulin molecule. It is, however, not known as yet whether the immunoglobulin protein component is homogeneous as in the case of amyloid fibrils of immunoglobulin origin.

Although the majority of the cases shown to have this class of amyloid protein have been obtained from patients with associated inflammatory diseases (e.g., rheumatoid arthritis), one of the patients was not known to have an associated inflammatory disorder. Conversely, it has been demonstrated that the major fibril protein obtained from one patient having amyloidosis associated with two distinct inflammatory diseases was of immunoglobulin origin. So far the presence of the amyloid protein of unknown origin (AUO) has not been demonstrated chemically in cases in which the major fibril protein is of immunoglobulin origin. It is possible that not until an antiserum is obtained to the AUO protein can a judgement be made as to whether it is a constituent of all amyloid fibril preparations. Therefore, although it is tempting to speculate that all amyloid fibrils are constituted in differing proportions of both immunoglobulin and "nonimmunoglobulin" protein, for the present it would seem advisable not to assume that all cases even of inflammation-associated amyloidosis will invariably be associated with this AUO protein as either a major or minor component of the fibril. The similarity, however, in both the clinical and pathologic manifestations of "primary" and "secondary" amyloidosis suggests that a similar, but not necessarily identical, pathologic process occurs in both.

Synthetic amyloid fibrils: It has been shown that polymeric aggregates of the variable region of some Bence Jones as well as poly-l-lysine in its β-conformation have the polarization properties of Congo red-stained amyloid fibrils. Insulin and glucagon, proteins with strikingly different primary structures, can be polymerized to form parallel and anti-parallel β-pleated sheet fibrils, respectively, as defined by infrared spectroscopic and x-ray crystallographic analysis. Under appropriate conditions of polymerization the fibrils formed are indistinguishable from those seen in many cases of amyloidosis. These protein polymers have polarization optical properties of Congo red-stained amyloid fibrils over a wide range of induced variation in their ultrastructural morphology. Prostatic corpora amylacea, which have similar properties but are composed of concentrically arranged fibrils, have recently been demonstrated to have x-ray diffraction features consistent with a β-structure. The presence of the β-pleated sheet as the major conformation, therefore, may be the underlying feature that distinguishes proteins having the properties of Congo red-stained amyloid fibrils from those, e.g. fibrin, that do not. We conclude that the terms "amyloid" and "amyloidosis" are generic terms. That is, fibrillar deposits of proteins having a β-conformation, regardless of chemical composition, will be classified as "amyloid" when Congo red birefringence is used as a defining property. The "amyloid" associated with insulinomas and the pancreatic islets in diabetes may be derived, for example, from insulin. (with Drs. Bladen, Eanes and Termine)

Honors and Awards: Member, Deutsche Akademie der Naturforscher
Leopoldina, 1973
Past-President, Histochemical Society, 1972-73

Publications:

Glenner, G.G., Harada, M., and Isersky, C.: The purification of amyloid fibril proteins. *Prep. Biochem*. 2: 39-51, 1972.

Glenner, G.G.: The pathogenetic and therapeutic implications of the discovery of the immunoglobulin origin of amyloid fibrils. *Hum. Path*. 3: 157-162, 1972.

Page, D.L., and Glenner, G.G.: Social interaction and wounding in the genesis of "spontaneous" murine amyloidosis. *Amer. J. Path*. 67: 555-570, 1972.

Termine, J.D., Eanes, E.D., Ein, D., and Glenner, G.G.: Infrared spectroscopy of human amyloid fibrils and immunoglobulin proteins. *Biopolymers* 11: 1103-1113, 1972.

Ein, D., Kimura, S., Terry, W.D., Magnotta, J., and Glenner, G.G.: Amino acid sequence of an amyloid fibril protein of unknown origin. *J. Biol. Chem*. 247: 5653-5655, 1972.

Glenner, G.G.: The discovery of the immunoglobulin origin of amyloid fibrils and its pathogenetic significance. *Acta path. Microbiol. Scand*. 80: 114-121, 1972.

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Serial No. NIAMDD-LEP-10
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cytogenetics

Principal Investigator: Dr. J. H. Tjio

Other Investigator: Dr. B. J. White

Cooperating Units: Maryland Psychiatric Research Center
Dr. W. W. Smith (NCI-LPHY)
Dr. G. Brecher (Univ. of California Medical Center, San Francisco, Calif.)

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Cytogenetic studies on the effect of LSD: Our double-blind, controlled study on the effects of the hallucinogens LSD and DPT on human chromosomes has not yet been completed because of problems arising from the death of Dr. W. N. Pahnke, the principal collaborator.

Chromosome identification and studies of chromosomal abnormalities in the mouse and human using the differential staining techniques of quinacrine mustard and Giemsa-banding.

Banding patterns in lymphocytic cell lines: Because of technical difficulties, the proposal to examine the human long-term lymphocyte cell lines maintained by Dr. A. Bloom (Ann Arbor, Mich.) has not been pursued.

Studies of mouse chromosomes using differential staining: Normal mouse chromosomes from several different strains, including AKR/J, CBA/J, CBA/HN, DBA, Balb/c, and AL/N have been examined with the Giemsa-banding technique. Only very minor karyotypic differences among these strains have been observed; the banding patterns have been remarkably consistent, the only changes being in the length of some secondary constrictions (which appear as negative-staining regions). The only exception to this was found in the AL/N strain, which was discovered to be uniformly homozygous for the T1Wh chromosome.

Also, the chromosomes of a wild mouse subspecies, called Kyushu (maintained by Dr. Michael Potter, NCI-LCGBY), are being investigated. These mice have a new immunoglobulin heavy chain linkage group which can best be explained as a recombinant of immunoglobulin genotypes, resulting from an unequal crossover between two linkage groups. Karyotypically the Kyushu mouse resembles a normal laboratory mouse except for chromosomes #1 and #2, which appear up to 1/5 longer than normal. The extra segment has been proven by C-banding to be composed of constitutive heterochromatin associated with the centromere. Studies of Kyushu/normal mouse hybrids are under way; we hope it will be possible to correlate the immunologic and karyotypic findings.

Chromosome identification in cell fusion experiments: Chinese hamster ovary cells (grown by Dr. T. T. Puck, University of Colorado Medical Center, Denver) with a specific enzymatic deficiency which results in glycine auxotrophy were fused with human cells; the resulting hybrids were grown on selective media to isolate those lines which had altered their glycine response. The chromosomes of these clones, and of the parent hamster line, are being examined in our laboratory to determine whether the hybrids retain any human chromosomes, and, if so, which ones are involved. If any human chromosomes are present, analysis of several clones should permit the assignment of the enzyme in question to the chromosome responsible for its production; linkage studies and gene mapping could follow.

Pattern of recovery of hemopoiesis in the mouse after irradiation and mechanical depopulation: The T1Ald mouse strain represents a Robertsonian fusion of two chromosome pairs and is derived from the AKR parent strain. It lends itself ideally to the production of radiation-induced chimaeras in which 97-100% of the bone marrow is of T1Ald karyotype, while the bone itself is of the AKR type. This system was used in cooperation with Dr. W. W. Smith (NCI-LPHY) and Dr. George Brecher (University of California Medical Center, San Francisco) to investigate the possible role of a common precursor cell of both bone and marrow following repopulation of a mechanically depopulated mouse femur. After lethal irradiation of the AKR host, T1Ald marrow was transplanted; subsequently, one femur of the recipient was mechanically depopulated. While "local" regeneration occurred, the marrow cells were of donor (T1Ald) type rather than host (AKR) type. Thus in this system the derivation of bone marrow from the postulated common precursor cell of the bone and marrow can be excluded, since such a cell would be of host origin.

In other experiments with the same system, the previous experience with T6T6 markers was confirmed, that marrow transplants do not "take" in normal unirradiated recipients, even though the genetic constitution of the AKR and T1Ald mice are presumably identical. Experiments are now planned to explore various possibilities to explain this phenomenon. Possibly, loss of chromosome material during fusion accounts for a minute difference and consequent immunologic incompatibility, which is nullified by irradiation. Another, more intriguing, possibility is that marrow stem cells are not in cycle and, although they may be seeded in normal recipients, proliferate only in irradiated recipients in which various hemopoietins or other factors stimulate the stem cell proliferation. These alternatives are now under investigation.

Clinical and cytogenetic studies on human congenital and developmental disorders: The differential staining methods of quinacrine mustard fluorescence and Giemsa-banding are being applied in our laboratory to studies of patients with various genetic disorders, abnormalities of gonadal development and function, and selected cases of infertility in the male. Since in many such patients no gross chromosomal abnormalities are detectable using routine cytogenetic methods, the differential staining procedures provide a means to detect small structural changes in chromosomes. A rearrangement that does not produce an obvious change in chromosome morphology can often be identified by altered banding patterns in the chromosomes involved, such as the duplication or deletion of chromosomal segments, peri-centric inversion, and some cases of reciprocal translocation. Since the Y chromosome has a characteristic intensely fluorescent region and the X chromosome has a characteristic Giemsa-banding pattern, differential-staining analysis of these chromosomes enables us to make a more precise diagnosis in many patients with abnormal gonadal development and function.

Publications:

White, B.J., Tjio, J.H., Van de Water, L.C., and Crandall, C.: Studies of mice with a balanced complement of 36 chromosomes derived from F_1 hybrids of T1Wh and T1Ald translocation homozygotes. Proc. Nat. Acad. Sci., U.S.A. 69: 2757-2761, 1972.

White, B.J., Tjio, J.H., Van de Water, L.C., and Crandall, C.: Trisomy for the smallest autosome of the mouse and identification of the T1Wh translocation chromosome. Cytogenetics 11: 363-378, 1972.

Serial No. NIAMDD-LEP-11
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cytogenetic Studies

Principal Investigator: Dr. B. J. White

Other Investigators: Dr. J. H. Tjio

Cooperating Units: Dr. E. Driscoll (NIDR-OMS)
Dr. R. Sherins (NICHD-RR)
Dr. J. Vaitukaitis (NICHD-RR)

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Cytogenetic and reproductive studies of mice with Robertsonian translocations and their hybrids.

Continued studies of the T1Wh strain: This strain, developed in our laboratory in 1967, is in generation 23 of inbreeding. The average litter size at birth has remained stable (6.0), and no abnormal characteristics have appeared. During the past year, the T1Wh translocation was identified in the NIH AL/N colony. This was established by Giemsa-banding of mitotic chromosomes of the AL/N and our T1Wh strain and by meiotic studies of AL/N X T1Wh F_1 hybrids. A paper concerning these findings has been submitted for publication.

Meiotic and other studies of mouse hybrids: Studies of the F_1 hybrids from crosses between T1Wh and T163H translocation homozygotes were continued. In an initial article, the incidence of trisomy 19 at birth in crosses between such (T1Wh x T163H) F_1 hybrids was described. The meiotic abnormalities responsible for nondisjunction of chromosome 19 were also studied. F_1 hybrids were then tested in other types of crosses to study the relationship of trisomy 19 to specific malformations (cleft palate) and to compare characteristics of normal and trisomic fetuses. A large number of crosses (134) were terminated between 12-1/2 days of gestation and term in order to examine, weigh, and karyotype trisomic fetuses and their littermates. Placentas and abnormal implantations were also studied, and certain organs were weighed and sectioned. This work is now completed, and a manuscript concerning the following findings is in preparation:

(1) Trisomy 19 is more frequent in crosses interrupted early in gestation (12-1/2 - 15-1/2 days) than in the same type of cross terminated later (16-1/2 days - term). Implantation studies showed many necrotic fetuses and reabsorptions, so the falling incidence of trisomy near term is probably related to continued in utero death of some trisomics.

(2) In all types of crosses, post-implantation losses of corpora lutea exceeded pre-implantation losses. This suggests that the majority of aneuploid embryos survive implantation.

(3) The incidence of trisomy 19 (27.2%) in a cross where survival of abnormal implants was expected to be maximal approached the maximum incidence expected from previous meiotic studies (32.8%), confirming the more accurate predictive value of direct meiotic studies in comparison to theoretical models of segregation.

(4) Male (T1Wh x T163H) F_1 hybrids had more trisomic progeny than hybrid females when crossed with normal mice. Since ovulation rates and pre- and post-implantation losses of corpora lutea were similar in both types of crosses (F_1 male x normal female and normal male x F_1 female), this indicates that nondisjunction is more frequent in male than female hybrids.

(5) Mean body and placental weights of viable trisomic fetuses are significantly lower than those of normal littermates at all stages between 12-1/2 days of gestation and birth. This phenomenon is clearly seen in parallel growth curves constructed from the weight data. The mean percentage of normal body weight for trisomics was 73.0 and for their placentas was 79.6.

(6) Mean weights of trisomic and normal lung and brain at term were significantly different. However, their relationship to mean body weight at term was similar, conforming to an earlier observation that trisomy 19 results in uniform reduction of size of all organ systems which is in proportion to the reduction of body weight.

(7) The only consistent specific malformation of trisomic fetuses was cleft palate. Since its incidence was higher (57.1%) in certain trisomics from crosses between hybrids and from crosses between hybrids and one parental strain (T163H) than in crosses between hybrids and normal mice (1.5%) and the other parental strain (T1Wh, 1.6%) specific genetic background affects its appearance.

(8) Sections of trisomic organs showed no specific or consistent abnormalities. Germ cells were present in both ovary and testis of trisomics, and there was no consistent gonadal pathology.

Previous reports from this laboratory described reduction of the mouse chromosome number from 40 to 38 and then from 38 to 36 by crossing mice with specific Robertsonian translocations. The strain with 36 chromosomes established last year (T1Ald/T1Ald T1Wh/T1Wh) has developed no abnormal characteristics and continues to be fully fertile. It is now being crossed with another strain (T1IEM) to determine if the mouse chromosome number can be further reduced to 34. This is part of a long-term study of the genetic and evolutionary significance of Robertsonian translocations.

Cytogenetic and dermatoglyphic studies of patients: A total of 35 patients with a variety of genetic or congenital abnormaliiies were karyotyped using the new differential staining techniques; dermatoglyphic studies were done in certain cases. These patients included gonadal dysgenesis (9 cases), Prader-Willi syndrome (3 cases), possible Klinefelter's syndrome (6 cases), multiple familial adenomatosis (3 cases), Schwachman-Diamond syndrome, athyrotic cretinism, intestinal lymphangiectasia, and familial vitamin D-resistant rickets.

The following variations from normal were found:

Gonadal dysgenesis	46,XXq-
Gonadal dysgenesis	45,X/46,XXr
Atypical Down's syndrome	47,XY 21+
Atypical Klinefelter's syndrome	47,XXY
Prader-Willi syndrome	46,XX 14p-
Familial vitamin D-resistant rickets	46,XY 21p+var

Only two of eight cases of gonadal dysgenesis in phenotypic females had abnormal karyotypes. Since gonadal dysgenesis is usually associated with a 45,X or 45,X/46,XX karyotype, our findings probably reflect referral of less typical cases. The relationship of dermatoglyphic features to karyotype in gonadal dysgenesis is of continuing interest, and collection of this information will continue.

A preliminary quinacrine-mustard staining study of sperm from infertile males with oligospermia or abnormal sperm morphology has been completed (in cooperation with Dr. R. Sherins, RR-NICHD). Of 18 patients sampled, two showed frequent double Y-chromatin bodies (9 and 18%); these samples had very abnormal sperm morphology. Since double Y-bodies can reflect nondisjunction of the Y at second meiotic metaphase in those with a normal XY karyotype or nondisjunction at first meiotic metaphase in those with an XYY karyotype, peripheral blood karyotypes will be necessary to follow up these two samples. Fourteen samples showed no double Y-bodies in the sperm, and two samples had one double Y-body (1%). These findings indicate that, except for very abnormal smears, the incidence of double Y-bodies is very low or zero in this group of patients. This is an even lower figure than that reported by others for normals (2%), and is more consistent with the low incidence of XYY males observed in the newborn male population (1/975).

Cytogenetic studies on the effect of Diazepam (Valium) on human chromosomes: Collection of samples for this study carried out with the cooperation of Dr. E. Driscoll (NIDR-OMS) is nearly complete, and analysis of slides and results should be available in the near future.

Publications:

White, B.J., Tjio, J.H., Van de Water, L.C., Crandall, C.: Studies of mice with a balanced complement of 36 chromosomes derived from F_1 hybrids of T1Wh and T1Ald translocation homozygotes. Proc. Nat. Acad. Sci., U.S.A. 69: 2757-2761, 1972.

White, B.J., Tjio, J.H., Van de Water, L.C., and Crandall, C.: Trisomy for the smallest autosome of the mouse and identification of the T1Wh translocation chromosome. Cytogenetics 11: 363-378, 1972.

Taussig, L.M., Braunstein, G.D., White, B.J., and Christiansen, R.L.: Silver-Russell dwarfism and cystic fibrosis in a twin. Amer. J. Dis. Child. 125: 495-503, 1973.

Serial No. NIAMDD-LEP-12
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972, through June 30, 1973

Project Title: Histopathologic, serum enzyme and other changes produced in animals by various environmental and other stresses.

Previous Serial Number: NIAMD-LEP-2

Principal Investigator: Benjamin Highman

Other Investigators: Drs. P. D. Altland, M. P. Dieter, H. M. Maling, W. H. Cyr, and R. P. Streett, Jr.

Cooperating Units: LPB-NIAMDD (Altland, Dieter)
LCP-NHLI (Maling)
Radiation Pathology Branch, AFIP (Cyr and Streett)

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

The projects discussed in last years report have been completed and terminated due to the retirement of Dr. Highman.

Publications:

Altland, P. D., Highman, B., and Dieter, M. P.: Reduced hypoxic tolerance of cold-acclimated rats: Serum enzyme and tissue changes. Am. J. Physiol. Vol. 222, 1441-1445, 1972

Highman, B., Cyr, W. H., and Streett, R. P., Jr.: Bacterial Endocarditis in X-irradiated Rats: Effect of Isoproterenol and Adrenergic Receptor Blocking Agents. Res. Comm. Chem. Path. Pharm. Vol. 5: 311-318, 1973.

Highman, B., and Altland, P. D.: Effect of Exercise and Training on Susceptibility of Epinephrine-Treated Rats to Baterial Endocarditis. Life Sciences Vol. 11, 931-939, 1972.

Highman, B.: Pathologic Changes Induced by Thorotrast. A Study Set with Syllabus and 90 Lantern Slides. American Registry of Pathology, AFIP p. 1-21, 1972.

ANNUAL REPORT
LABORATORY OF CHEMICAL BIOLOGY
NIAMDD

The Laboratory of Chemical Biology has maintained its interests in research activities on the nature of the relationships between structure and function in proteins, the regulation of protein biosynthesis, synthetic and immunologic approaches to the mechanisms of polypeptide chain folding and, more recently, the beginnings of some work on the isolation and study of human interferon.

I. Relationships between protein sequence and three-dimensional structure

A. Studies employing complementation of peptide fragments prepared from staphylococcal nuclease

The kinetics of formation of Nuclease-T' from Nuclease-T-(6-48) and Nuclease-T-(49-149) was studied by measuring intensity of tryptophan fluorescence as a function of time. The kinetic behavior fits first order kinetics. The presence of the ligands and the change of pH from 5 to 8 had no effect on the rate constant. As reported in previous years, another type of complementation, the interaction of Nuclease-(1-126) and Nuclease-T-(49-149), simultaneously yields, in 1 min, two alternative ordered complexes, Type I and II, both resembling nuclease. Type I, analogous to Nuclease-T, has an ordered structure formed by the segment of residues 1 to 48 of Nuclease-1(1-126) and the entire amino acid sequence of Nuclease-T-(49-149). The ordered structure of Type II consists of essentially the entire amino acid sequence of Nuclease-(1-126) and the carboxyl terminal part of Nuclease-T-(49-149). Labelled Nuclease-T', ((6-48) +[1-^{14}C]acetyl-(50-149)) was incubated with unlabelled Nuclease-T-(50-149) in the presence of thymidine-diphosphate (pdTp) and Ca^{2+} at pH 8. The rate of exchange between [1-^{14}C]acetyl-(50-149) incorporated into Nuclease-T' and free labelled (50-149) was measured as a function of temperature. Appreciable exchange was observed only above 25°. Activation enthalpy of the unfolding of Nuclease-T', as measured by the exchange experiments in the presence of ligands, was 50 kcal. The rate of exchange in the presence of 1.5 mM of pdTp was approximately one-third of that in the presence of 0.3 mM of pdTp. Since the population of liganded Nuclease-T should not change appreciably at these two different concentrations of pdTp, the effect of concentration of pdTp on the apparent rate of unfolding of Nuclease-T, as indicated by the rate of the exchange, may suggest that the unfolding occurs only after pdTp dissociates from Nuclease-T.

In general, the kinetic and equilibrium experiments with complementing peptide confirm our previous conclusion that the stabilization of three-dimensional structure is an extremely cooperative phenomenon and that most of the amino acid units of the chain must be present to permit the formation of the native conformation. In many cases the presence of ligands causes marked stabilization and occasionally can even induce the formation of native-like structure in fragments of the protein sequence considerably shorter than the entire molecule. For example, it was found that apocytochrome *c* can bind with a disordered heme-containing fragment containing residues 1-65 prepared by the method of Harbury *et al*. to form a product with spectral properties closely resembling those of native cytochrome *c*. Similarly, a large fragment of serum albumin was capable of assuming a sufficiently native-like structure to bind to long chain fatty acids immobilized on Agarose. (Taniuchi, Bohnert, Light, Peters, Fisher)

B. Immunological approaches to the determination of conformation equilibria in staphylococcal nuclease

It has been postulated, from work in this laboratory, that certain parts of protein chains which, in the native protein exist in essentially self-sufficient, cooperatively stabilized structures, may occupy such conformations for extremely short periods of time as a result of what might be termed "flickering" equilibria between the native and disordered forms. If this were the case, antibodies prepared against the entire native protein should contain sub-populations of gamma globulins that would recognize various individual antigenic determinants in the structure. It has been possible to isolate such sub-populations using affinity chromatography of anti-nuclease sera on Sepharose columns bearing attached peptide fragments of nuclease. These antibody fractions inhibit nuclease activity. By measuring the ability of the relevant peptide fragments, predominantly disordered in solution, to prevent the inhibition of native nuclease activity by the antibody, one may estimate the fraction of the peptide fragment which at any given moment is occupying a conformation that is recognized by the antibody to that portion of the native structure. In these experiments it is assumed that the fragment shuttles from disordered to native-like states and that there is simultaneously an equilibrium involving association and dissociation of the properly folding fragment and the antibodies directed against it. Using such an approach, it has been found that the portion of the nuclease sequence extending from residue 99-149 appears to occupy the native "format" about 0.02 percent of the time. This approach is being continued with relation to other parts of the nuclease molecule in an effort to determine the kinetics of the various individual equilibria that may form the basis for the nucleation process in protein folding. (Furie, Schechter, Eastlake, Sachs)

C. Nuclear magnetic resonance studies of proteins

The availability of a 220 MHz NMR apparatus with sophisticated Fourier adaptation and Varian time averaging has made possible a number of NMR studies on proteins of interest to this laboratory. Titration curves for the imidazole C2 protons of the four histidine residues of nuclease have been determined. It has been found that the addition of calcium ions increase the pKa of three of these residues but lowers that of the active site histidine. Control studies of the effects of ionic strength on these pKa values show that the occupancy of calcium ions in the active site makes it 0.4 pH units more difficult to protonate the neighboring histidine residue. The addition of thymidine diphosphate (pdTp), a substrate analogue, increases the calcium effect but has no further action on the NMR curves. These results are being used to calculate the effective dielectric constant of the active site. The metal binding site of nuclease was also studied (at pH 7.0) using lanthanide ions. The data suggest that the lanthanide ions bind tightly to the Ca(II) binding site of nuclease and enhance the binding of substrate analogue to nuclease; they do not promote catalytic activity against DNA substrate. The perturbation of the four imidazole C2 proton resonances in the 220 MHz NMR spectrum of nuclease by lanthanide ions bound to the metal binding site of nuclease has been examined to determine the distance from the bound metal to the histidine residues in solution. For lanthanide shift ions [Eu(III)] the chemical shift of an observed resonance by a rare earth metal is a function of $1/r^3$, where r is the distance between the observed residue and the metal. Assuming the angular dependence term is unity, the relative distances between each of the four histidines and the metal binding site was found to be within 5% of the relative distances calculated from the crystallographic model. Using this same approach, it has been possible to reassess the geometry and solution structure of the active site of nuclease using pdTp, 3' TMP, and 5' TMP. The susceptibility of the resolvable proton and phosphorus resonances of the substrate analogues to broadening by paramagnetic GD(III) and DY(III) can be correlated to absolute distances. Seven distinct resonances have been identified for pdTp and TMP, and each appears to be selectively perturbed. Control experiments have demonstrated that the broadening of each resonance is due to the effect of a single bound paramagnetic ion and is due neither to substrate-enzyme binding or substrate-metal complex formation alone.

The imidazole-C2 protons of the four histidine residues of bovine pancreatic ribonuclease A have also been resolved, assigned to the corresponding amino acids in the sequence, and titration curves of chemical shift vs pH constructed for each resonance. The acid perturbations in three of these titration curves have been shown to be due to interactions with neighboring carboxyl groups on the basis of previous studies from this laboratory with model histidyl compounds and computer curve fitting of mathematical models for interacting groups. An alkaline perturbation in the titration curve for histidine 12 has been shown to be due to interaction with lysine 41. The effects of anions, especially phosphate and sulfate ions, and of various substrate analogues, including especially the dinucleoside phosphonate, UpcA, on these titration curves have also been studied. The NMR properties of the protons and phosphorous nucleus of the substrate analogue when complexed to ribonuclease A and S have also been examined. These results indicate that there is no direct histidine-phosphate interaction in the complex, that there is little strain at the phosphorous nucleus, and that histidine 12 may act as both a base and an acid in the first step in the cleavage of RNA. The possibility of a proton transfer by water molecules is also suggested by the results. (Schechter, Furie, Griffin, East, Anfinsen, Eastlake)

D. Synthetic studies of protein structure and function

Perhaps the most ideal way of studying relationships between structure and function of proteins is by total or partial synthesis of the molecule with the substitution of normally occurring amino acids by other amino acids or amino acid analogues. Two major avenues have been taken. The first involves the synthesis of the fragment (6-47) of staphylococcal nuclease with various substitutions, particularly of charged amino acids in the active center region of the enzyme with other amino acids bearing shorter or longer side chains of different charges. The results confirm the conclusions drawn from examination of the crystallographic model, in the sense that four uniquely placed carboxyl groups seem to be involved in the binding of the essential calcium ion and that certain positively charged amino acids interact with the phosphate groups of substrate or inhibitor. A second avenue has been the use of semisynthetic derivatives of staphylococcal nuclease and ribonuclease S (which contain complementing fragments, residues 1-20 and 21-124). For example, the replacement of the phenylalanine residue at position 8 of ribonuclease S with ^{13}C-enriched phenylalanine has made it possible to demonstrate, by high resolution NMR spectroscopy, that the disordered 1-20 fragment assumes its typically helical form upon complexing with the larger 1-124 fragment. This kind of study typifies current experiments which aim to use this combination of synthetic and NMR procedures to carry out a detailed investigation of the contribution of individual amino acid residues and even of individual atoms within each residue in the formation and stabilization of three-dimensional structure. (Chaiken, Griffin, Dunn, DiBello).

E. Kinetic studies of the mechanism of action of staphylococcal nuclease

The synthesis of analogues of nuclease described above has been complemented by direct kinetic studies of substrate digestion by the enzyme. A spectrophotometric assay was utilized to follow the kinetics of catalysis by staphylococcal nuclease. Equilibrium dialysis was carried out in micro cells with $^{45}Ca^{2+}$ and ^{14}C-pdTp under a variety of conditions of concentration. The ^{14}C-pdTp was synthesized and purified in this laboratory. The pH dependence of the kinetics of staph. nuclease catalysis has been quantitated and has yielded constants which will aid in elucidation of critical functional groups. Binding of Ca^{2+} and pdTp to the nuclease has been shown to be an interdependent process with the presence of the second ligand increasing the binding of the first ligand by a factor of roughly 20 fold. We have demonstrated the binding involves one molecule of both ligands under the normal conditions of assay (pH 8.8). At higher pH values where tyrosine side chains are expected to ionize it appears that up to three Ca^{2+} atoms are binding. (Chaiken, DiBello, East)

II. Improvements in the solid phase synthesis of polypeptides

Because of the general interest in the laboratory in the synthesis of protein analogues, efforts have been made to improve the yields in this method and to speed up the synthetic process. The rapid modification of the solid phase method has been applied to the synthesis of a large fragment of staphylococcal nuclease, containing residues 114-145. Residue 140, which is normally tryptophan in the native protein, was replaced with phenylalanine during the synthesis since this helps eliminate side reactions during assembly of the chain and since the tryptophan residue is known to be unessential to activity. The final product was removed from the solid support and is at present under purification by ion exchange chromatography and affinity chromatography. The latter procedure is applicable because the fragment under consideration complements with residues 1-126 of nuclease to form a noncovalently stabilized complex with enzymic activity. Thus, 1-126, immobilized on Sepharose, may be used to catch the "good" synthetic material. Former methods of solid-phase synthesis required at least four days for the synthesis of bradykinin while the present technique permits completion in about 5 hours. If this method can be extended to other peptides, such as the 32 residue peptide discussed above, it should enable investigators to study many more problems by this method than could previously have been studied in a comparable time. (Sachs, Corley, Anfinsen)

III. New projects on the structure and function of several protein systems

A. The structure and function of the histidyl t-RNA synthetase from *Salmonella typhimurium*

The histidyl-tRNA synthetase from *Salmonella typhimurium* is particularly valuable because so much is known about the genetics and biochemistry of this histidine operon, particularly from work in NIAMDD by Ames, Martin, Goldberger and their colleagues. We have begun a detailed study of the structure and function of this enzyme. The pure enzyme has been studied by chemical means, including amino acid analyses, N-terminal amino acid determination, and peptide maps, and physical means, including analytical ultracentrifugation and electrophoresis. The interaction with ligands has been studied by fluorescence quenching, equilibrium dialysis and other means. It has been found that the binding of ATP and t-RNA quench the intrinsic fluorescence of the protein significantly; the binding of histidine has a small effect; the binding of Mg^{++} has no effect. Where appropriate these properties have been used to measure the binding constant and stoichiometry of the interaction of the ligands to the protein. These results are being checked by equilibrium dialysis and ultraviolet difference spectroscopy, and these parameters are being studied as a function of temperature, pH, and the concentration of other ligands. Antobodies to the enzyme have been prepared. *Salmonella* organisms with mutant histidyl t-RNA synthetase enzymes are now available and the properties of these proteins are being studied. These findings represent the initial steps in the study of the structure and mechanism of this protein. The protein is important in the mechanism of biosythesis of proteins in the cell and, possibly, in regulatory mechanisms. Because of the extensive knowledge of the histidine operon in *Salmonella*, this protein offers an unusual opportunity to correlate structure and function with genetics and physiology. Further, the interaction of the protein with a nucleic acid, a nucleotide, an amino acid, and a divalent metal should allow clarification of the general nature of such protein interactions. Thus it is anticipated that specific and general inferences may be made about the role of the t-RNA synthetases in the molecular biology of cells. (Schechter, Di Natale, Schiller, Eastlake)

B. Isolation, structural determination, and synthesis of human interferon

The availability of fairly large amounts of crude human interferon from NIAID, through a commercial contract involving the use of human leukocyte cell cultures challenged with Newcastle virus, makes it feasible to consider the isolation of pure agent for subsequent structural and biological study. Eventual synthesis of human interferon would appear to be the only practical approach to the use of the substance clinically since it is present in such minute amounts in biological sources. The crude culture fluid has been exposed to a variety of pH and denaturing conditions and to gel filtration, ion exchange chromatography, affinity chromatography on lecten columns, and electrofocusing. Human interferon is stable for a long period to 0.1 M acetic acid, pH 3, at room temperature and has been purified extensively by three passes of dialyzed, lyophilized crude material through G100 Sephadex. The best material obtained so far has been injected into goats with the hopes of producing an antibody that might be used for solid phase immunoabsorption. Such a step would eliminate the tedious and wasteful early procedures used to date. (Anfinsen, Corley, Goldberger)

C. Studies of complex formation between bovine neurophysin and the neurohypophysial peptide hormones, oxytocin and [8-lysine] vasopressin

Oxytocin and [8-lysine] vasopressin are nonapeptide hormones which are stored in neurosecretory granules of the mammalian posterior pituitary gland in the form of hormone-protein complexes. The proteins which bind these hormones in neurosecretory granules are the neurophysins, a small class of proteins which have recently been isolated in pure form and sequenced. Studies of the isolated neurophysin-hormone complexes may contribute to our understanding of the structure of the hormone-macromolecule complex as it exists in the neurosecretory granules. The structure and microenvironment of various aromatic amino acid residues in the neurophysin-hormone complexes and in the isolated molecules were characterized by absorbance, CD, and PMR spectroscopy. The various spectral results were correlated to show that the second and third amino acid residues of the hormone molecules are rigidly bound to the neurophysin molecule in a hydrophobic environment. It was also shown, using both hormones and tripeptide analogs of the hormone's three N-terminal residues, that tyrosine-49 of neurophysin undergoes a conformational change upon complex formation such that its local environment changes from hydrophobic to hydrophilic. The chemical synthesis of hormones specifically enriched in carbon-13 will be undertaken in order to explore the possible uses of carbon-13 nuclear magnetic resonance for the study of hormone structure and hormone binding processes. It is hoped that this development of new methods for the study of hormone-protein interactions may be of general applicability. (Griffin)

D. Isolation and characterization of unique protein components of Paramecium

Work on the isolation of the trichocysts of Paramecium continues. These components of the Paramecium cell surface are of particular interest because of their extremely interesting contractile properties, which may serve as valuable models for the understanding of similar protein systems in higher organisms. A valuable by-product of these studies is the isolation of an invertebrate hemoglobin from Paramecium. (Steers, Pollack)

IV. Studies on the mechanism of repression of the histidine operon of *Salmonella typhimurium*

Most of the work of the Section of Biosynthesis and Control has been focused on the mechanism of repression of the histidine operon of *Salmonella typhimurium*. Previous studies over the past few years had brought to light the fact that the enzyme encoded in the first structural gene of the operon -- tthe *G* enzyme -- is involved in regulation of the operon. This autogenous type of regulation, in which the protein specified by one of the genes of an operon plays a role in regulating expression of that very operon, has now been found by other workers in several other regulated systems.

It has been known for a long time that aminoacylated histidine tRNA is required for repression of the histidine operon. Our studies suggested that this regulatory molecule may function as a complex formed by interaction with the *G* enzyme. Studies on the interaction between histidine tRNA and *G* enzyme *in vitro* have now shown that the enzyme has a very high affinity for tRNA, that it binds histidine tRNA in preference to any other species of tRNA, and that it binds the aminoacylated form of histidine tRNA in preference to deacylated tRNA^{His}.

A *cis*/*trans* test of the effect of the *G* enzyme on regulation of the histidine operon has shown that the enzyme exerts its regulatory effect not by acting locally, at its site of synthesis, but by acting as a freely diffusible gene product, as is the case for other known regulatory proteins.

Several mutants have now been isolated in which a mutation in the *hisG* gene not only causes an alteration in the structure of the *G* enzyme but also causes constitutive expression of the histidine operon.

Using DNA of a defective Ø80 transducing phage carrying the histidine operon(Ø80d*his*), we have been able to show that the purified *G* enzyme interacts specifically with the bacterial portion of the phage genome, suggesting that it interacts with some regulatory element of the histidine operon.

To test the functional equivalent of this binding of the enzyme to the DNA of Ø80d*his*, a purified system was developed for transcribing DNA *in vitro*. Using the phage DNA as template, we could show that histidine operon messenger RNA is synthesized *in vitro* from the "sense" strand, and this RNA was found to sediment in sucrose density gradients at 38S, the known value for histidine operon-specific messenger RNA synthesized *in vivo*. When purified *G* enzyme was added to the *in vitro* transcription system, there was a specific and severe inhibition of transcription of the histidine operon, indicating that the enzyme acts as a negative control element.

Studies on regulation of amino acid biosynthesis in mammalian cells have not yet brought to light any specific repression or induction under conditions in which the end product of the biosynthetic pathways was limiting or in excess.

(Goldberger, Kronenberg, Vogel, Meyers, Deeley, Lipson)

Serial No. NIAMDD-LCB-1
1. Lab. of Chemical Biology
2. Protein Conformation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the relationship between the amino acid sequences and the three-dimensional structure of protein: the mechanism of folding of protein

Previous Serial Number: NIAMD-LCB-1, 2 and 3

Principal Investigator: Hiroshi Taniuchi

Other Investigators: Janice L. Bohnert
Albert L. Light

Man Years:

Total: 2.2
Professional: 1.1
Other: 1.1

Project Description:

Objectives: To find the mechanism of folding of protein on the basis of the amino acid sequence

A Working Hypothesis: A model concerning the mechanism of folding of protein is developed on the basis of the previous findings described below (No. 1 to 4, see the previous reports).

1. The three-dimensional structure of nuclease involves a dynamic equilibrium of folding and unfolding under physiological conditions.

2. The complete amino acid sequence is required for the formation of stable structures of nuclease and ribonuclease A.

3. The nuclease polypeptide chain can be cleaved at only a few points without disrupting the unique distribution of forces stabilizing the native conformation. The inclusion of a redundant portion of the amino acid sequence does not interfere with the orderly folding of two complementing but overlapping fragments.

4. There is doubtless a relationship between the stabilization of the native conformation and the conformational cooperativity of protein and that an incomplete and disordered polypeptide chain has a greatly diminished conformational cooperativity. For example, while the values for intrinsic viscosities of nuclease and Nuclease-T are the same (2.4±0.2 g/ml), those for fragments of residues 1 to 126 and of residues 49 to 149 are at the level of 10.0 g/ml. The hydrodynamic volume of RNase having two or more incorrectly paired disulfide bonds is greater than native RNase.

If it is assumed that only the complete amino acid sequence provides the combination of forces which are responsible for the unique three-dimensional structure and stabilization of the native conformation, then the cleavage of a 'non-permissible' peptide bond or the removal of an essential segment of the polypeptide chain would not only alter the state of local side chain interactions but also disrupt the unique distribution of forces. Then, the effect would be transmitted throughout the three-dimensional structure, resulting in disorder of the polypeptide chain. It is therfore hypothesized that the specific energy state stabilizing the native conformation of nuclease cannot be formed until the conformation of disordered nuclease changes to one similar to that of the native protein. In other words, it is assumed that the folding of disordered nuclease into the native-like conformation can occur statistically with little change in the conformational energy and that the native conformation would then be stabilized by the development of the unique state of cooperative interactions throughout the three-dimensional structure having the 'proper' distribution of polar and non-polar side chains. On the other hand, although disordered Nuclease-(1-126) (fragment of residues (1 to 126)) could also fold similarly into the native-like conformation, the stabilization of the native conformation of Nuclease-(1-126) would not occur. Two similar native conformations of intact nuclease having higher and lower energies, therefore, arise from this model - an activated and a ground state, respectively. Thus, the model would visualize that the native structure of nuclease is a statistical distribution of conformational states trapped by the energy barrier suppressing the transition from the ground to the activated state.

In order to test the hypothesis, the system of complementation of two overlapping fragments were used. As reported in previous years, the interaction of Nuclease-(1-126) and Nuclease-T-(49-149) simultaneously yields, in 1 min, two alternative ordered complexes, Type I and II, both resembling nuclease. Type I, analogous to Nuclease-T, has an ordered structure formed by the segment of residues 1 to 48 of Nuclease-(1-126) and the entire amino acid sequence of Nuclease-T-(49-149). The ordered structure of Type II consists of essentially the entire amino acid sequence of Nuclease-(1-126) and the carboxyl terminal part of Nuclease-T-(49-149). Type I has a greater heat stability than that of Type II. The model assumes a ground state and an activated state for each ordered complex and a finite number of non-native states representing the disordered complexes formed from the two fragments. The rate of formation of an ordered complex is assumed to depend only on the conformational relationship between the non-native states and the activated state and is similar for both ordered complexes. Therefore, the populations of the two ordered complexes formed from the two fragments in 1 min are expected to be approximately equal to each other regardless of the difference in their thermodynamic stabilities. The final distribution of the two ordered complexes should be determined by their thermodynamic stabilities, if the system is allowed to reach equilibrium through unfolding and refolding.

Methods Employed and Major Findings: Labelled Nuclease-T', ((6-48)+[1-^{14}C]acetyl-(50-149)) (see the report of last year) was incubated with unlabelled Nuclease-T-(50-149) (P_3) in the presence of thymidine-diphosphate (pdTp) and Ca^{2+} at pH 8. The rate of exchange between [1-^{14}C]acetyl-(50-149) (labelled P_3) incorporated into Nuclease-T' and free P_3 was measured as a function of temperature by the method reported last year. In order to separate Nuclease-T' and P_3 from the mixture of the two containing ligands, a column having two packed zones, Sepharose-nuclease over Sepharose-pdTp, was used. When the mixture was applied to the column, pdTp was first absorbed by Sepharose-nuclease so that the subsequent absorption of Nuclease-T' to Sepharose-pdTp was not hindered. The exchange between incorporated P_3 and free P_3 was strongly suppressed in the presence of ligands. Appreciable exchange was observed only above 25°. Activation enthalpy of the unfolding of Nuclease-T', as measured by the exchange experiments in the presence of ligands, was 50 kcal. The rate of exchange in the presence of 1.5 mM of pdTp was approximately one-third of that in the presence of 0.3 mM of pdTp. Since the population of liganded Nuclease-T should not change appreciably at these two different concentrations of pdTp, the effect of concentration of pdTp on the apparent rate of unfolding of Nuclease-T, as indicated by the rate of the exchange, may suggest that the unfolding occurs only after pdTp dissociates from Nuclease-T. Indeed, thermal transition of nuclease and Nuclease-T, as measured by change of ellipticity at 222 nm, also shifted toward higher temperature at a higher concentration of pdTp, being consistent with the pdTp-concentration dependence of unfolding.

Nuclease-(1-126) and Nuclease-T-(49-149) was mixed at pH 8. The ratio of the populations of type I and II formed was estimated on the basis of the quantity of isolated fragment residues 111 to 149. The latter was obtained from the mixture after digestion with trypsin in the presence of pdTp and Ca^{2+} by the method described in the previous report. When the redistribution of type I and II through unfolding and refolding was prevented by addition of ligands at 23° or by incubation for a short time (4 min) at 6°, the populations of the two complexes formed was approximately equal. On the other hand, when the system was allowed to reach an equilibrium state, the ratio of the population of type I to type II was approximately 2 to 1. The results are consistent with the expectation by the hypothesis.

As a step toward the crystallization and eventual X-ray diffraction study of type II complex, the crystalization of nuclease after trifluoroacetylation and deblocking was performed in order to test the effect of the treatment on the formation of crystal. For, the same treatment is used in the preparation of Nuclease-(1-126) (see the previous report). The treated nuclease yielded crystals microscopically indistinguishable from those of untreated nuclease.

The kinetics of formation of Nuclease-T' from Nuclease-T-(6-48) and Nuclease-T-(49-149) was studied by measuring intensity of tryptophan fluorescence as a function of time (see the report of last year). The kinetic behavior fits first order, rather than second order, kinetics. The first order rate constant of the formation of Nuclease-T is in the range of 0.03 to 0.05 sec^{-1}. The change of temperature from 5° to 45° caused only a small increase in the rate of formation of Nuclease-T. The presence of the ligands and the change of pH from 5 to 8 had no effect on the rate constant. The complementations of type II and of fragment residues 1 to 126 and P_3 also fit first order kinetics and show rate constants equivalent to that of Nuclease-T. (This paragraph is made on the basis of the manuscript by A. Light, H. Taniuchi and R.F. Chen.)

The properties of porphyrin-cytochrome c and apocytochrome c in solution were compared with those of native cytochrome c by the study of their viscosity and of their circular dichroism and fluorescence. Porphyrin-apo-cytochrome *c* appears to have a compact structure similar to native cytochrome *c*, though it is less stable to heat denaturation. Apo-cytochrome *c* has the properties of an enlarged and disordered structure (see the last year report). However, apo-cytochrome *c* can bind with the disordered heme-containing fragment of residues 1 to 65, prepared by the method of Corradin and Harbury and forms a product with spectral properties closely resembling native cytochrome *c* in the same manner as does the COOH-terminal fragment of residues 66 to 104 (Corradin and Harbury (1971) Proc. Nat. Acad. Sci. U.S.A., 68, 3036). The observations indicate that the presence of the porphyrin ring covalently bonded with cysteines 14 and 17 is essential for cytochrome *c* to exist in the stable native conformation. (This paragraph is from the paper by W.R. Fisher, H. Taniuchi and C.B. Anfinsen.)

Significance to Biomedical Research: Many investigators are applying potential functions of non-bonded interactions to calculate the energies of polypeptide conformations. It is assumed that the individual forces involved are additive and that the atomic coordinate of a protein minimizing the calculated energy representes the native structure. On the other hand, in order to take into account the cooperativity of conformational change of a protein, other investigators treat the subject by statistical mechanics. Here, it is assumed that individual nonbonded interactions are equivalent and affected uniformly by change of a parameter. Our observations described above appear to suggest that the situation of the nonbonded interactions forming and stabilizing the native structure of a protein is more complicated than those assumed by these theories. There appears to be a unit of cooperative three-dimensional structure in which forces work not simply additively but cooperatively throughout the three-dimensional structure. However, individual, that is local, interactions in the three-dimensional structure are apparently not equivalent in relation to the stabilization of the three-dimensional structure. A fuller understanding of such non-bonded interactions is important, since the biological system is crucially regulated and maintained by these non-bonded interactions.

Proposed Course of Project:

1) Elucidation of the three-dimensional structure of crystalline Nuclease-T' by X-ray diffraction study.

2) Crystallization of type II complex (see above) to be used for X-ray diffraction study.

3) Crystallization and X-ray diffraction study of semi-synthetic Nuclease-T' containing synthetic-Nuclease-T-(6-48) and native Nuclease-T-(49-149) with the collaboration of Dr. C.B. Anfinsen, Section on Protein Chemistry.

4) Study on the kinetics and thermodynamics involving the interaction of complementing fragments of nuclease, ribonuclease A, cytochrome *c* and other pertinent proteins.

Publications:

Peters, T., Jr., Taniuchi, H. and Anfinsen, C.B.: Affinity chromatography of serum albumin with fatty acids immobilized on Agarose. J. Biol. Chem. 248, 2447, 1973.

Fisher, W.R., Taniuchi, H. and Anfinsen, C.B.: On the role of the heme in the formation of the structure of cytochrome c. J. Biol. Chem. 248, in press (1973).

Taniuchi, H.: The dynamic equilibrium of folding and unfolding of Nuclease-T'. J. Biol. Chem. 248, in press (1973).

Serial No. NIAMDD-LCB-2
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Preparation and studies of native and semisynthetic non-covalent protein complexes

Previous Serial Number: NIAMD-LCB-4 and 5

Principal Investigator: Irwin M. Chaiken

Other investigators: Guillermo R. Sánchez
John H. Griffin
Ben M. Dunn

Cooperating Units: 1) Laboratory of Chemistry, National Heart and Lung Institute, NIH (Edward A. Sokolosky).
2) Physical Sciences Laboratory, Divison of Computer Research and Technology, NIH (Jack S. Cohen).
3) Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada (Murray H. Freedman and James R. Lyerla, Jr.).

Man Years:

Total: 1.5
Professional: 1.5

Project Description:

1) To prepare, by chemical synthesis, polypeptides and protein fragments which interact with corresponding native polypeptides or protein fragments to form biologically active complexes.
2) To obtain purified synthetic products suitable for detailed study as part of semisynthetic protein complexes.
3) To use the semisynthetic complexes for studying the conformational and functional properties of the particular systems in question, notably by studies of synthetic analogues.
4) To use such semisynthetic complexes also as models for investigating the application of various new kinds of techniques for studying proteins and interacting protein systems.
5) To obtain native components of interacting protein-peptide systems for characterization of naturally occurring species.

Methods Employed: Several systems have been investigated, including bovine pancreatic ribonuclease-S' and the neurophysin-hypophyseal peptide hormone complexes. In the former case, a specific protein fragment, ribonuclease-

S-(1-20) has been studied with respect to the noncovalent complex formed upon addition to the complementary fragment ribonuclease-S-(21-124). Besides the native complex (obtained from native bovine pancreatic ribonuclease A), semisynthetic derivatives have been obtained by prepapation of synthetic ribonuclease-S-(1-20) analogues.

For the interacting systems of the neurophysins with the peptide hormones oxytocin and vasopressin, again the native noncovalent complexes are being studied after isolation of the neurophysins from posterior pituitary gland (bovine and other). Synthesis of oxytocin and vasopressin derivatives also is being carried out.

Major Findinds: Analogues of semisynthetic ribonuclease-S' have been made for active site and conformationally important residues in the (1-20) fragment. A major effort has been made to prepare ^{13}C-enriched and ^{19}F-containing synthetic fragments which have been used to study the interaction of (1-20) fragments with ribonuclease-S-(21-124) by carbon 13 and fluorine 19 nuclear magnetic resonance spectroscopy. It has been shown that such selective labelling can be achieved synthetically and that the products provide valuable tools for measuring structural parameters for specific residues in going from disordered (denatured) to ordered (native) environments. The techniques involved also have indicated the application to the study of the mechanism of action of ribonuclease-A and -S.

At the present stage, the isolation of the bovine neurophysins is just being completed. Further studies are in the preliminary stage.

Significance to Biomedical Research: The above studies allow the description of structural bases for conformation and biological function for the specific proteins in question. Conclusions for the specific cases of study are applicable to an understanding of other proteins as well. Thus, these studies contribute to the goal of understanding the underlying principles of normal and abnormal protein function.

Proposed Course of Action:

1) Continuance of projects for semisynthetic ribonuclease-S (and Staphylococcal nuclease-T'), with specific attention to important synthetic analogues for which functional and conformational information will lead to an increased understanding for these proteins in particular and for other proteins in general.

2) Study of neurophysin-peptide hormone systems, with special attention to chemical characterization of the noncovalent complexes, to the biosynthetic origin of these complexes, and to the biological function of these in higher vertebrates.

3) Use of the above systems as models for investigating the applicability of various techniques, including several types of nuclear magnetic resonance spectroscopy, for studying proteins in general.

Publications:

Chaiken, I.M.: Studies with semisynthetic noncovalent protein complexes, In Meienhofer, J. (ed.): Proc. Third Amer. Peptide Symp. Ann Arbor Science Pub., 1972. p. 255.

Freedman, M.H., Lyerla, J.R., Chaiken, I.M. and Cohen, J.S.: Carbon 13 nuclear magnetic resonance studies on selected amino acids, peptides and proteins. European J. Biochem. 32, 215, 1973.

Chaiken, I.M., Freedman, M.H., Lyerla, J.R. and Cohen, J.S.: Preparation and studies of ^{19}F- and enriched ^{13}C-labelled semisynthetic ribonuclease-S' analogues. J. Biol. Chem. 248, 884, 1973.

Chaiken, I.M.: Chemical studies of structural features in staphylococcal nuclease-T', J. Biol. Chem. 247, 1999, 1972.

Chaiken, I.M. and Sánchez, G.R.: Active site properties of [aspartic acid 43]-semisynthetic nuclease-T'. J. Biol. Chem. 247, 6743, 1972.

Sánchez, G.R., Chaiken, I.M. and Anfinsen, C.B.: Structure-function relationships at the active site of nuclease-T'. J. Biol. Chem. 247, 1999, 1972.

Serial No. NIAMDD-LCB-3
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Semisynthetic analogues of Ribonuclease S'.

Previous Serial Number: NIAMD-LCB-4

Principal Investigator: Ben M. Dunn

Other Investigators: Irwin M. Chaiken
Carlo Di Bello

Cooperating Units: Laboratory of Chemistry, NIAMDD (Louis Cohen)

Man Years:

Total: 1.5
Professional: 1.5

Project Description:

Objectives:

1) To examine the relationship between functional group character and catalytic activity in a model protein.

2) To develop methods for functional purification of semisynthetic derivatives.

3) To examine the influence of side chain character on stability of tertiary structure and peptide-protein interactions.

Methods Employed: Solid phase peptide synthesis has been utilized to introduce amino acids with altered properties into the sequence of the peptide that complexes with the native fragment of ribonuclease, residues 21-124, to produce a semisynthetic complex. The small peptide of 15 residues will form an active complex with 21-124 if the native sequence of 1-15 is used. We have introduced the following variations. At position 12 of the 1-15 peptide we have added 4-fluoro-L-Histidine in place of the naturally occurring histidine. We have also added the 1-methyl-L-histidine and 3-methyl-L-histidine derivatives. At position 9 of the sequence we have substituted glycine or leucine for the native glutamic acid.

We have used sulfoethyl Sephadex ion exchange chromatography to effect the purification of the crude synthetic material, followed by Sephadex gel filtration to obtain the purified peptide. The peptides are then characterized by activity assays, inhibition curves, difference spectroscopy, and other probes of structural integrity with respect to their ability to form functional complexes with the native 21-124 fragment.

Major Findings: By mixing the crude solid phase derived 1-15 peptides with native 21-124 a complex is formed between synthetic peptides of proper sequence and the 21-124 fragment. Sulfoethyl sephadex chromatography under controlled conditions of pH and ionic strength is then sufficient to separate the stable complex from the synthetic peptide of incorrect sequence. Additional steps of gel filtration yield the synthetic peptide isolated from the 21-124 native fragment as confirmed by amino acid composition.

The 4-fluoro-L-histidine-12-synthetic(1-15) peptide forms a stable semisynthetic complex as evidenced by chromatography, inhibition of native 1-20 binding to 21-124 (K_i for synthetic peptide determined as 2.2×10^7 M), difference spectroscopy, thermal stability, and resistance to proteolysis. The complex is without catalytic activity against two ribonuclease substrates. This lack of activity may be correlated with the reduction of the pK_a of the imidazole side chain from 6.5 in the native histidine to 2.5 in the 4-fluorohistidine. Thus the basic strength of the imidazole at position 12 seems to determine the catalytic efficiency in the ribonuclease system.

The methylated derivatives, 1-methyl-histidine 12-synthetic(1-15) and 3-methyl-histidine 13-synthetic(1-15) are without activity in the crude state. We have obtained some evidence from affinity chromatography for binding of the 3-methyl derivative to the 21-124 fragment and we plan to continue purification of these derivatives.

The peptides with alterations in the side chain at position 9 have been shown to yield catalytically active complexes when mixed with native 21-124. This property has been exploited to achieve purification of the crude material resulting in production of purified 1-15 peptides with side chain modification in position 9. We are currently characterizing these peptides with respect to the quantitative strength of interaction with the native 21-124. These studies will aid in elucidating the crucial question of the organization of polypeptides into functional tertiary structure.

Significance to Biomedical Research: A deeper understanding of the factors that govern catalytic activity and the formation of tertiary structure is indispensible to the eventual understanding of the molecular basis of disease. The design of improved or corrected proteins must be based on an understanding of structure-function relationships in protein chemistry.

Proposed Course of Project:

1) Further characterization of the peptides already in hand with respect to interactions with the 21-124 fragment.

2) Design, synthesis, purification, and characterization of other derivatives in this series.

3) Correlation of functional properties with side chain characteristics.

Serial No. NIAMDD-LCB-4
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Kinetic studies of the mechanism of action of staphylococcal nuclease.

Previous Serial Number: NIAMD-LCB-6

Principal Investigator: Ben M. Dunn

Other Investigators: Irwin M. Chaiken
Carlo DiBello
David East
Christian B. Anfinsen

Cooperating Units: Heuristics Laboratory, Division of Computer Research and Technology, NIH (Richard Schrager)

Man Years:

Total: 0.5
Professional: 0.5

Project Description:

Objectives:
1) To quantitate the steady-state kinetic parameters for hydrolysis of deoxythymidine 5'-p-nitrophenylphosphate 3'-phosphate by staphylococcal nuclease as a function of pH.
2) To characterize the binding of deoxythymidine-3',5'-diphosphate (pdTp) and Ca^{2+} to staphylococcal nuclease by equilibrium dialysis.

Methods Employed: A spectrophotometric assay described in detail in the previous report was utilized to follow the kinetics of catalysis by staphylococcal nuclease. Equilibrium dialysis was carried out in micro cells with $^{45}Ca^{2+}$ and ^{14}C-pdTp under a variety of conditions of concentration. The ^{14}C-pdTp was synthesized and purified in this laboratory.

Major Findings: The pH dependence of the kinetics of staph. nuclease catalysis has been quantitated and has yielded constants which will aid in elucidation of critical functional groups. Binding of Ca^{2+} can pdTp to the nuclease has been shown to be an interdependent process with the presence of the second ligand increasing the binding of the first ligand by a factor of roughly 20 fold. We have demonstrated the binding involves one molecule of both ligands under the normal conditions of assay (pH 8.8). At higher pH values where tyrosine side chains are expected to ionize it appears that up to three Ca^{2+} atoms are binding.

Significance to Biomedical Research: Understanding the principles of catalysis by macromolecules will allow a deeper understanding into fundamental life processes. Clearer appreciation of the relationship between structure and function will enable rational decisions to be made concerning correction of defects in molecular diseases.

Proposed Course of Project:

1) Continued probind into the catalytic events of staph. nuclease reactions using rapid reaction techniques.

2) Extension of the equilibrium dialysis studies to derivatives of staph. nuclease arising from synthesis of polypeptides and modification of the native molecule.

Publication:

Dunn, B.M., DiBello, C. and Anfinsen, C.B.: The pH-dependence of the steady-state kinetic parameters for staphylococcal nuclease catalyzed hydrolysis of deoxythymidine-3'-phosphate-5'-p-nitrophenylphosphate in H_2O and D_2O. J. Biol. Chem., in press.

Serial No. NIAMDD-LCB-5
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunological properties of staphylococcal nuclease: determination of conformational equilibria.

Previous Serial Number: NIAMD-LCB-8

Principal Investigators: Bruce Furie
Alan N. Schechter
Christian B. Anfinsen
Ann Eastlake

Cooperating Units: Laboratory of Immunology, NCI (David H. Sachs)

Man Years:

Total: 1.3
Professional: 0.9
Other: 0.4

Project Description:

Objectives: It has been shown in this laboratory that the interaction of antibodies with nuclease and fragments from this protein may be used to quantitate the conformational equilibria of polypeptide chains in solution. This has been accomplished by the preparation of antibodies to whole nuclease and fragments, the purification of non-precipitating antibodies to single antigenic regions in the carboxy-terminal end of the protein by sequential affinity chromatography and the quantitation of the interaction of these antibodies with antigens, singly and in various combinations. It is the purpose of the current work to extend these studies to the properties of other regions of this protein, to determine a conformational equilibrium parameter for the entire protein, to study further the effects of the interaction with antibodies on the activity and complementation of nuclease, and to apply these methods to other proteins.

Methods Employed: Polypeptide fragments from nuclease have been prepared and purified and antibodies to these and native nuclease have been produced in goats. The antisera have been purified by selective immunoadsorption on Sepharose columns to which proteolytic fragments of nuclease have been covalently bound. By these means antibodies to single antigenic determinants have been prepared which are immuno-specific and non-precipitating, but which inactivate nuclease.

Carbamylation, primarily of α-NH_2 groups, by $KN^{14}CO$ has been used to label nuclease and its fragments so that direct equilibrium and kinetic measurements of the interaction with antibody could be made. The labeled polypeptides behave similarly to the native ones. A double precipitation method using rabbit anti-goat gamma globulin and scintillation counting has been developed for this purpose.

Major Results: As discussed previously, a non-precipitating, monospecific antibody against region 99-126 has been prepared. This inactivates the enzyme and it has been possible to measure the on-rate, off-rate, and equilibrium constant of the interaction of the antibody with nuclease. Competition of this reaction with fragment 99-149 of nuclease has allowed the determination of the $k_{conf.}$ (2.4×10^{-4}) for the equilibrium between its disordered forms and the "native format" conformation similar to that which exists for this sequence in the native molecule. Other physical techniques have not been able to determine such values. Complementation of this fragment with others has been found to increase markedly the value of $k_{conf.}$.

We have now prepared non-precipitating antibodies specific for the "random" conformation of fragment 99-126. Antisera to fragment 99-149 were purified by immunoabsorption to Sepharose columns of nuclease and fragment 127-149. The $k_{assoc.}$ of the interaction of anti-$(99\text{-}126)_r$ with fragment 99-149 has been estimated at about 10^7 M by double precipitation. The degree of competition for this reaction by ^{14}C labelled nuclease allows a determination of $k_{conf.}$ of 3×10^3 for native nuclease. This indicates that about 1 of 3000 molecules of nuclease is in an unfolded conformation with respect to the antigenic determinant at any time. In the presence of ligands (calcium ions and thymidine diphosphate), a $K_{conf.}$ of 1.5×10^4 was estimated. These results are consistent with the stabilization of the native conformation by ligands.

The separation of several other proteolytic fragments of nuclease has been accomplished and antibodies specific for them are being prepared by analogous means.

The 0.2% residual activity of fragment 1-126 has been shown to be inherent to the fragment and not due to contaminating nuclease from the finding that anti-$(127\text{-}149)_n$ inactivates nuclease but not fragment 1-126.

Significance to Biomedical Research: There are many naturally occurring proteins which are not easily studied in their intact state, such as membrane proteins, lipoproteins, and multiple-subunit proteins. Since conformation often plays a major role in the biologic properties of such proteins, one needs means of assessing the conformational properties of components or subunits of these proteins. Because such fragments and subunits may contain only a very small percentage of the corresponding native conformation, there are often no physical methods capable of such assessment. The immunologic analysis of a $K_{conf.}$ therefore provides a parameter of conformation unobtainable by physical methods alone. The method described herein represents a general approach for the determination of $K_{conf.}$ of any polypeptide chain. Requirements include a labelled polypeptide chain, a nonprecipitating antibody which distinguishes between the random or native conformation, and an association constant for the interaction of antibody and its corresponding antigen. The technique may therefore prove valuable in elucidating the structure and dynamics of more complicated protein systems.

Proposed Course of Project: The examination of $K_{conf.}$ for nuclease under a variety of conditions (pH, temperature, ligands) will continue. Additionally, efforts to determine $K_{conf.}$ of other polypeptide fragments and to isolate "activating" antibodies will be pursued.

The immunologic approach to the study of the conformational equilibria of other proteins will be developed. We are presently preparing antibodies to several forms of Hb S with the intent of examining the factors which shift the conformational equilibria of Hb S toward the native or toward the aggregated forms.

Publications:

Sachs, D.H., Schechter, A.N., Eastlake, A. and Anfinsen, C.B.: Antibodies to a distinct antigenic determinant of staphylococcal nuclease. J. Immunol. 109, 1300-1310, 1972.

Sachs, D.H., Schechter, A.N., Eastlake, A. and Anfinsen, C.B.: Inactivation of staphylococcal nuclease by the binding of antibodies to a single antigenic determinant. Biochemistry 11, 4268-4273, 1972.

Sachs, D.H., Schechter, A.N., Eastlake, A. and Anfinsen, C.B.: An immunological approach to the conformational equilibria of polypeptides. Proc. Nat. Acad. Sci. U.S.A. 69, 3790-3794, 1972.

Serial No. NIAMDD-LCB-6
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Nuclear magnetic resonance studies of the structure and function of staphylococcal nuclease.

Previous Serial Number: NIAMD-LCB-10 and 11

Principal Investigators: Alan N. Schechter
Bruce Furie
John H. Griffin
David A. East
Christian B. Anfinsen
Ann Eastlake

Cooperating Units: Physical Sciences Laboratory, DCRT (Jack S. Cohen)
Department of Chemistry, Laurentian University, Sudbury, Canada (Evert Nieboer)

Man Years:

Total: 1.8
Professional: 1.5
Other: 0.3

Project Description:

Objectives: Although much has been learned of the solution properties of staphylococcal nuclease, the mechanism of enzymatic activity is still unclear. The X-ray diffraction determination of the structure has not given detailed information about the geometry of the active site, the exact orientation of the substrate, or the electronic properties of the residues in the active site. High resolution nuclear magnetic resonance spectroscopy allows inferences about the environments and effective pKa values of groups in the protein. Perturbation of the active site protons by the calcium ion cofactor or substrate analogs may be used to study the effects of these on the protein structure. The paramagnetic lanthanide ions may be used to measure distances in the macromolecule by their effects on proton chemical shift and line widths. It is the object of this project to study the geometry of the active site of staphylococcal nuclease by these methods.

Methods Employed: Nuclease has been purified to homogeneity. Enzyme activity, chemical modification, difference spectroscopy, and susceptibility to proteolysis have been studied by previously described methods.

Solutions of nuclease at 10% concentration in 100% D_2O-0.1 M NaCl have been used for nuclear magnetic resonance studies on the Varian 220 MHz spectrometer. Spectra have been rec orded on a time averaging computer or with a Fourier transform computer. Chemical shift and line width data have been determined from the spectra by manual or computer analysis.

Major Results: Titration curves for the imidazole C2 protons of the four histidine residues of nuclease have been determined. It has been found that the addition of calcium ions increase the pKa of three of these residues but lowers that of the active site histidine. Control studies of the effects of ionic strength on these pKa values show that the occupancy of calcium ions in the active site makes it 0.4 pH units more difficult to protonate the neighboring histidine residue. The addition of thymidine diphosphate (TDP) a substrate analogue increases the calcium effect but has no further action on the NMR curves. These results are being used to calculate the effective dielectric constant of the active site.

The metal binding site of nuclease was also studied (at pH 7.0) using lanthanide ions. The binding of Gd(III) or Nd(III) to nuclease was found to be associated with tyrosine perturbations in the UV spectrum. These residues include tyrosine 85 and tyrosine 115 as inferred from studies using nitrotyrosine-85-nuclease and nitrotyrosine-115-nuclease. A binding constant of 9 μM was estimated by spectrophotometric titration. The Ca(II) required for the binding of nuclease to TDP-Sepharose could be replaced by Nd(III). Nd(III) also stabilized the nuclease structure to trypsin digestion, in the presence or absence of TDP. None of the lanthanide ions replaced Ca(II) in the generation of nuclease activity toward DNA under the conditions employed. Kinetic data compatible with a model of competitive inhibition of Ca(II) by La(III), Pr(IIII), Nd(III), Gd(III), Dy(III), and Yb(III) yielded inhibition constants of 1-2 μM. These data suggest that the lanthanide ions bind tightly to the Ca(II) binding site of nuclease and enhance the binding of substrate analogue to nuclease; they do not promote catalytic activity against DNA substrate.

The perturbation of the four imidazole C2 proton resonances in the 220 MHz NMR spectrum of nuclease by lanthanide ions bound to the metal binding site of nuclease has been examined to determine the distance from the bound metal to the histidine residues in solution. For lanthanide shift ions [Eu(III)] the chemical shift of an observed resonance by a rare earth metal is a function of $1/r^3$, where r is the distance between the observed residue and the metal. Assuming the angular dependence term is unity, the relative distances between each of the four histidines and the metal binding site was found to be within 5% of the relative distances calculated from the crystallographic model. The stoichiometry for the metal:nuclease complex was 1:1. For lanthanide broadening reagents [Gd(III) or Dy(III)], the broadening of the observed resonance by the rare earth metal is a function of $1/r^6$. Using this relationship the relative distances between each of the four histidine resonances and the bound Gd(III) or Dy(III) were within 10% of the relative distances determined from the crystallographic model. From these studies, it would appear that the solution structure of nuclease is similar to the crystal structure. The use of paramagnetic lanthanide ions as NMR probes of the metal binding site thus permits the measurement of intraprotein distances in solution and appears to be an alternative to crystal analysis in the study of some aspects of three-dimensional structure.

Using this same approach, it has been possible to reassess the geometry and solution structure of the active site of nuclease using 3',5' TDP, 3' TMP, and 5' TMP. The susceptibility of the resolvable proton and phosphorus resonances of the substrate analogues to broadening by paramagnetic Gd(III) and

Dy(III) can be correlated to absolute distances. Seven distinct resonances have been identified for TDP and TMP, and each appears to be selectively perturbed. Control experiments have demonstrated that the broadening of each resonance is due to the effect of a single bound paramagnetic ion, and is due neither to substrate-enzyme binding or substrate-metal complex formation alone.

Significance to Biomedical Research: The high resolution NMR studies on the effects on the histidine residues of calcium ions and TDP give information about the effective pKa values, dielectric constant, and geometry of the active site of nuclease. The study of the effects of lanthanide ions on these histidine resonances gives information confirming the similarity of the crystal and solution studies and the validity of the approach using paramagnetic ions. The detailed geometrical inferences that may be made from understanding the effects of lanthanide ions on protons (and other nuclei) on the substrate analogue should allow a visualization of the active site with extreme precision. This information will effectively complement that of X-ray diffraction in understanding the structure and mechanism of this enzyme.

These methods should be applicable to other metal binding proteins, especially the biologically important calcium proteins, where studies have been hindered by the lack of useful electronic or magnetic properties of calcium ions.

Proposed Course of the Project: We plan to complete studies on the protons of the protein and substrate analogue, study the phosphorus nuclei of the analogues and measure the correlation time, $_c$, by determining the relaxation time T_1 as a function of frequency. These studies will be extended to the natural abundance ^{13}C NMR spectrum of the substrate analogues.

A kinetic analysis of the possible hydrolysis of nitrophenyl-TDP by nuclease in the presence of lanthanide ions is in progress, as is a study by NMR of true substrates in the inactive nuclease-lanthanide-nucleic acid complex.

Publications:

Schechter, A.N.: Chemical and physical factors involved in protein folding as exemplified by staphylococcal nuclease: Part II. Kinetics. PAABS Revista 1, 461-464, 1972.

Schechter, A.N.: Factores quimicos y fisicos involucrados en el plegamiento de las proteinas utilizando como ejemplo la nucleasa del estafilococo. Parte II. Cinetica. PAABS Revista 1, 465-468, 1972 (Reprints of previous papers, pp. 469-494).

Schechter, A.N.: Conformational transitions in staphylococcal nuclease. Proc. IV International Biophysics Congress, Moscow, 1972.

Nieboer, E., East, D., Cohen, J.S., Furie, B., and Schechter, A.N.: Proton magnetic resonance studies of rare-earth ion interactions with staphylococcal

nuclease. Proceedings of the Tenth Rare Earth Research Conference, 763-769 (1973).

Furie, B., Eastlake, A., Schechter, A.N. and Anfinsen, C.B.: The interaction of lanthanide ions with staphylococcal nuclease. J. Biol. Chem., in press.

Serial No. NIAMDD-LCB-7
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Nuclear magnetic resonance studies of proteins.

Previous Serial Number: NIAMD-LCB-7

Principal Investigators: Alan N. Schechter
John H. Griffin

Cooperating Unit: Physical Sciences Laboratory, DCRT (Jack S. Cohen)

Man Years:

Total: 0.6
Professional: 0.6

Project Description:

Objectives: Nuclear magnetic resonance techniques give information useful to understanding the structure of proteins in solution and the interactions of proteins with substrates and other ligands. At 220 MHz it is possible to resolve proton signals from the different aromatic residues and make inferences about the electronic environment of these residues in the three-dimensional structure of the protein. These inferences may be tested by studies of suitable chemical model compounds and by computation of the data with various mathmatical models. Studies of protein-substrate analogue complexes, with observations of protons in both species, may be used to make inferences about the geometry of the active site and the mechanism of enzyme activity. The development, at the NIH and elsewhere, of methods for studying the resonances of other nuclei (^{13}C, ^{31}P, etc.) and of Fourier transform analysis has further expanded the applicability of these methods to the study of macromolecular structure.

Methods Employed: The Varian XL-100 and HA-220 MHz nuclear magnetic spectrometers have been used. For most studies, protein samples have been exchanged with pure D_2O and residual protons have been measured and recorded on a Varian time averaging computer or Fourier transform computer. pH titrations have been accomplished by measuring time-averaged spectra at closely spaced pH values. Temperature, ionic strength, and the concentration of substrate analogs have been used as independent variables.

Many spectra have been digitized, recorded on magnetic tape, and then analyzed into their component Lorentzian curves with an IBM 360/50 computer and 2250 display so that accurate values of chemical shift, area, and peak width could be obtained. Theoretical titration curves of chemical shift vs. pH for the individual resonances have been computed for the Henderson-Hasselbalch relationship and for interacting titrating groups. These curves have been compared to our data by computer fitting.

Proteins have been modified by removal of ligands or prosthetic groups to study the effects of these factors on structure. Proton titrations in water have been done with ribonuclease to study certain slowly exchangeable groups. Recently, the phosphorous nucleus in certain inhibitors of ribonuclease have been observed with a ^{31}P probe.

Major Findings: As was discussed in the last report, the imidazole C2 protons of the four histidine residues of bovine pancreatic ribonuclease A have been resolved, assigned to the corresponding amino acids in the sequence, and titration curves of chemical shift *vs* pH constructed for each resonance. The acid perturbations in three of these titration curves have been shown to be due to interactions with neighboring carboxyl groups on the basis of previous studies from this laboratory with model histidyl compounds and computer curve fitting of mathematical models for interacting groups. An alkaline perturbation in the titration curve for histidine 12 has been shown to be due to interaction with lysine 41.

The titration curves of ribonuclease S have been studied in an analogous manner. In contrast to the findings of other workers, it has been found that these results are almost identical to those for ribonuclease A. This is consistent with studies by other techniques and indicates that inferences from the X-ray data for ribonuclease S are probably valid for understanding ribonuclease A.

The effects of anions, especially phosphate and sulfate ions, on these titration curves have been studied. These results show that the anions increase the imidazole pKa values but have little effect on the pKa of the acid perturbations. The total effect, however, is to increase the apparent asymmetry of the titration curve. These results confirm the explanation for the asymmetries outlined above and have further implications for the geometry of the active site.

The effects of various substrate analogues, including especially the dinucleoside phosphonate, UpcA, on these titration curves have been studied. The NMR properties of the protons and phosphorous nucleus of the substrate analogue when complexed to ribonuclease A and S have also been examined. These results indicate that there is no direct histidine-phosphate interaction in the complex, that there is little strain at the phosphorous nucleus, and that histidine 12 may act as both a base and an acid in the first step in the cleavage of RNA. The possibility of a proton transfer by water molecules is also suggested by the results.

It has been found that in H_2O ribonuclease A has one slowly exchanging proton in the downfield region. The titration behavior of this proton at several temperatures and in the presence of several substrate analogues has demonstrated that this proton may be assigned to one of the imidazole-NH groups of histidine 119. The detection and identification of this resonance gives another parameter in studies of the active site of this enzyme and in understanding the factors which affect exchange rates with solvent in proteins.

The studies of the titration curves of the histidine residues of sperm whale and horse heart myoglobin, discussed in this report last year, have been completed and published. The study of the titration curves of the histidine residues of cytochrome *c* and apo-cytochrome *c* has been completed. These results have been compared to other physical indices of structure to characterize the large effect of the prosthetic group on the structure and stability of this protein.

Significance to Biomedical Research: These methods allow the study of the behavior of different parts of a protein molecule in solution--thus combining advantages of the usual spectroscopic methods and X-ray crystallography. The results indicate that the imidazole proton titration curves are sensitive probes to electronic interactions within a protein. The studies with substrate analogues give signficant information about the geometry and electronics of the active site of ribonuclease; they also have implications for understanding the mechanism of catalysis. The existence of a slowly exchanging proton in the active site of ribonuclease offers important information about solvent accessibility. The data for myoglobin and cytochrome *c* indicates that nuclear magnetic resonance studies of histidine residues can give information about the effects of the heme prosthetic groups on the structure of these proteins.

Proposed Course of Project: The collection and analysis of NMR data on these and other proteins will continue in an attempt to enlarge our understanding of protein structure-function relations in solution.

Publications:

Schechter, A.N., Sachs, D.H., Heller, S.R., Shrager, R.I. and Cohen, J.S.: Nuclear magnetic resonance titration curves of histidine ring protons. III. Ribonuclease. *J. Mol. Biol*. 71, 39-48, 1972.

Cohen, J.S., Hagenmaier, H., Pollard, H. and Schechter, A.N.: Proton magnetic resonance study of the histidine residues of sperm whale and horse myoglobins. *J. Mol. Biol*. 71, 513-519, 1972.

Griffin, J.H., Schechter, A.N. and Cohen, J.S.: NMR study of the RNase-UpcA complex and its implications for the enzyme mechanism. *Ann. N.Y. Acad. Sci*., in press.

Griffin, J.H., Cohen, J.S. and Schechter, A.N.: The assignment of an exchangeable low field NH proton resonance of ribonuclease A to the active site histidine residue 119. *Biochemistry*, in press.

Cohen, J.S., Griffin, J.H. and Schechter, A.N.: Nuclear magnetic resonance titration curve of histidine ring protons. IV. The effects of phosphate and sulfate on ribonuclease. *J. Biol. Chem*., in press.

Serial No. NIAMDD-LCB-8
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The structure and function of the histidyl t-RNA synthetase from Salmonella typhimurium.

Previous Serial Number: None

Principal Investigators: Alan N. Schechter
Paola Di Natale
Peter Schiller
Ann Eastlake

Cooperating Unit: Istituto di Chimca Biologica, Universita di Napoli, Naples, Italy (Prof. Francesco De Lorenzo)

Man Years:

Total: 1.5
Professional: 1.2
Other: 0.3

Project Description:

Objectives: The t-RNA synthetase enzymes play a central role in the molecular biology of protein synthesis in the cell. They activate specific amino acids and then esterify these to specific transfer RNA species for use in messenger RNA directed protein synthesis. They interact, in these two reactions, with t-RNA, amino acid, ATP, and Mg^{++}. Thus they are ideal for studying protein-nucleic acid, protein-nucleotide, and protein-metal interactions. There is genetic evidence in several systems that these enzymes may act in a regulatory manner for transcription or translation. Thus, they may be important in cellular control mechanisms.

The histidyl t-RNA synthetase from Salmonella typhimurium is particularly valuable because so much is known about the genetics and biochemistry of this histidine operon, particularly from work in NIAMDD by Ames, Martin, Goldberger and their colleagues. We have begun a detailed study of the structure and function of this enzyme.

Methods Employed: Several large preparations of Salmonella bacteria were grown in the NIH fermenter unit. The cells were lyzed and an ammonium sulfate fraction prepared at the NIH using modifications of methods developed by DeLorenzo and Ames. The initial preparations of pure histidyl t-RNA synthetase were completed in the laboratory of Professor DeLorenzo in the University of Naples. More recently, the entire purification, using several types of chromatography, has been done at the NIH.

The pure enzyme has been studied by chemical means, including amino acid analyses, N-terminal amino acid determination, and peptide maps, and physical means, including analytical ultracentrifugation and electrophoresis. The interaction with ligands has been studied by fluorescence quenching, equilibrium dialysis, and other means.

In addition, the phenylalanyl t-RNA synthetase has been purified from these preparations because of the interesting fluorescent nucleotide in the corresponding t-RNA which should allow detailed study of this protein-nucleic acid interaction by energy transfer methods.

Major Findings: Several hundred mgm of the histidyl t-RNA synthetase have been purified. Analytical ultracentrifugation and disc gel electrophoresis has shown the enzyme to be pure. A molecular weight of 80,000 has been established by equilibrium centrifugation and gel chromatography. A subunit molecular weight of 40,000 was determined by centrifugation in guanidine hydrochloride and by SDS electrophoresis. Peptide maps suggest that the subunits are identical. The amino acid composition of the enzyme has been established.

The enzyme assay has been improved and steady state kinetic studies have been begun. It has been found that the binding of ATP and t-RNA quench the intrinsic fluorescence of the protein significantly; the binding of histidine has a small effect; the binding of Mg^{++} has no effect. Where appropriate these properties have been used to measure the binding constant and stoichiometry of the interaction of the ligands to the protein. These results are being checked by equilibrium dialysis and ultraviolet difference spectroscopy, and these parameters are being studied as a function of temperature, pH, and the concentration of other ligands.

Antibodies to the enzyme have been prepared. Salmonella organisms with mutant histidyl t-RNA synthetase enzymes are now available and the properties of these proteins are being studied.

The phenylalanyl t-RNA synthetase is now also suitable for detailed enzymological, chemical, and physical studies.

Significance to Biomedical Research: These findings represent the initial steps in the study of the structure and mechanism of this protein. The protein is important in the mechanism of biosynthesis of proteins in the cell and, possibly, in regulatory mechanisms. Because of the extensive knowledge of the histidine operon in Salmonella, this protein offers an unusual opportunity to correlate structure and function with genetics and physiology. Further, the interaction of the protein with a nucleic acid, a nucleotide, an amino acid, and a divalent metal should allow clarification of the general nature of such protein interactions. Thus it is anticipated that specific and general inferences may be made about the role of the t-RNA synthetases in the molecular biology of cells.

Proposed Course of Project: The structural analysis of the histidyl t-RNA synthetase enzyme will continue with the preparation of proteolytic fragments and sequence determination, in collaboration with W. Terry of NCI. The interaction of the enzyme with ligands will be studied by additional techniques, including circular dichroism, nuclear magnetic resonance, immunochemistry, and kinetics. A detailed analysis of the enzyme kinetics and mechanism will be performed. The properties of enzymes from mutant organisms will be studied.

The phenylalanyl enzyme will be studied analogously but with emphasis on the fluorescence properties of the enzyme and the t-RNA. Pure species of the corresponding t-RNA molecules will be prepared for these studies.

Significance to Biomedical Research: These findings represent the initial steps in the study of the structure and mechanism of this protein. The protein is important in the mechanism of biosynthesis of proteins in the cell and, possibly, in regulatory mechanisms. Because of the extensive knowledge of the histidine operon in *Salmonella*, this protein offers an unusual opportunity to correlate structure and function with genetics and physiology. Further, the interaction of the protein with a nucleic acid, a nucleotide, an amino acid, and a divalent metal should allow clarification of the general nature of such protein interactions. Thus it is anticipated that specific and general inferences may be made about the role of the t-RNA synthetases in the molecular biology of cells.

Proposed Course of Project: The structural analysis of the histidyl t-RNA synthetase enzyme will continue with the preparation of proteolytic fragments and sequence determination, in collaboration with W. Terry of NCI. The interaction of the enzyme with ligands will be studied by additional techniques, including circular dichroism, nuclear magnetic resonance, immunochemistry, and kinetics. A detailed analysis of the enzyme kinetics and mechanism will be performed. The properties of enzymes from mutant organisms will be studied.

The phenylalanyl enzyme will be studied analogously but with emphasis on the fluorescence properties of the enzyme and the t-RNA. Pure species of the corresponding t-RNA molecules will be prepared for these studies.

Serial No. NIAMDD-LCB-9
1. Lab. Chemical Biology
2. Protein Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies of complex formation between bovine neurophysin and the neurohypophysial peptide hormones, oxytocin and [8-lysine] vasopressin

Previous Serial Number: none

Principal Investigator: John H. Griffin

Cooperating Units: DCRT, LPS (Jack S. Cohen); French Atomic Energy Commission, Saclay, France (Paul Cohen, Robert Alazard)

Man Years:

Total: 0.3
Professional: 0.3

Project Description:

Objectives: Oxytocin and [8-lysine] vasopressin are nonapeptide hormones which are stored in neurosecretory granules of the mammalian posterior pituitary gland in the form of hormone-protein complexes. The proteins which bind these hormones in neurosecretory granules are the neurophysins, a small class of proteins which have recently been isolated in pure form and sequenced. Studies of the isolated neurophysin-hormone complexes may contribute to our understanding of the structure of the hormone-macromolecule complex as it exists in the neurosecretory granules. Moreover, the horomone-neurophysin complexes provide a useful model system for the study of the binding of peptide hormones by proteins as well as for the development of physical methods which may be valuable to characterize hormone binding processes.

Methods Employed: Absorbance and circular dichroism (CD) difference spectra were measured using a Cary model 15 spectrophotometer and a Jouan model II CD Dichrograph, respectively.

Proton nuclear magnetic resonance (PMR) spectra were obtained using the Varian 220 MHz spectrometer equipped for continuous wave as well as Fourier transform operation.

Major Findings: The structure and microenvironment of various aromatic amino acid residues in the neurophysin-hormone complexes and in the isolated molecules were characterized by absorbance, CD, and PMR spectroscopy. The various spectral results were correlated to show that the second and third amino acid residues of the hormone molecules are rigidly bound to the neurophysin molecule in a hydrophobic environment. It was also shown, using both hormones and tripeptide analogs of the hormone's three N-terminal residues, that tyrosine-49 of neurophysin undergoes a conformational change upon complex formation such that its local environment changes from hydrophobic to hydrophilic. Additionally, the temperature variation of $(1/T_2)$ of the hormone's aromatic amino acid proton resonances indicated that the kinetics of complex formation and breakdown are relatively rapid with the lifetime of the neurophysin-hormone complex being less than 10 milliseconds. The lone histidine residue of neurophysin-I has a microscopic pK_a value of 6.7 which is not substantially changed in the presence of bound hormone, thus suggesting histidine is not involved in complex formation.

The spectral fine structure in the absorbance and CD difference spectra between 250 and 350 nm were compared to each other in order to define the characteristics of tyrosyl CD perturbations. The neurophysin-hormone complex is a good model for such a purpose since it contains no tryptophan and only tyrosine-49 of neurophysin and tyrosine-2 of the hormone. Tripeptide analogs of the hormone in which phenylalanine-2 replaced the hormone's tyrosine-2 were also used to characterize the spectral properties of the neurophysin's tyrosine-49. The results showed that when tyrosine-2 is placed into a hydrophobic environment, as indicated by a red-shifted absorbance spectrum, the fine structure in the CD difference spectrum exhibits its 0-0 transition near 287 nm; and, conversely, when tyrosine-49 is placed into a hydrophilic environment, as indicated by a blue-shifted absorbance spectrum, the fine strucutre in the CD difference spectrum exhibits its 0-0 transition near 282 nm. These results provide the first clear demonstration in a model protein system that tyrosyl CD fine structure can be used to characterize the microenvironment of tyrosyl residues.

Significance to Biomedical Research: Characterization of the structure of the neurophysin-hormone complexes *in vitro* may further our understanding of the structure of these important hormone-protein complexes as they exist *in vivo*. A deeper understanding of the molecular basis for hormone recognition and binding by the neurophysin proteins may contribute to our developing concepts about the recognition of peptide hormones by hormone receptor molecules. Understanding the molecular basis for interactions between hormones and receptor molecules is an essential part of a molecular definition of the mechanism of hormone action.

Proposed Course of Project: The chemical synthesis of hormones specifically enriched in carbon-13 will be undertaken in collaboration with Irwin Chaiken (LCB) in order to explore the possible uses of carbon-13 nuclear magnetic resonance for the study of hormone structure and hormone binding processes. It is hoped that this development of new methods for the study of hormone-protein interactions may be of general applicability.

Serial No. NIAMDD-LCB-10
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Improvements in the solid-phase synthesis of polypeptides

Previous Serial Number: NIAMDD-LCB-9

Principal Investigators: David H. Sachs
Lila Corley
Christian B. Anfinsen

Man Years:

Total: 1
Professional: 0.4

Project Description:

Objectives: The times usually devoted to the required steps in the Merrifield solid-phase synthetic scheme do not permit the manual addition of more than about two or three amino acid residues per day. This means that considerable time is needed even for the synthesis of rather short polypeptides, as we have found in our previous syntheses of nuclease fragments. We have therefore set out to modify the synthetic scheme in order to increase the speed of synthesis and to minimize those losses attributable to the long reaction times under harsh conditions which are presently used in this method.

Methods Employed: Methods were developed for following the coupling and deblocking reactions during solid-phase synthesis in small funnels so that the time course could be determined. An apparatus was constructed permitting the rapid addition of reagents with mixing by bubbling rather than by rocking. The process of shrinking and swelling the resin in order to increase washing efficiency was studied and employed.

Major Findings: Since the report of June 30, 1972, the rapid modification of the solid phase method has been applied to the synthesis of a large fragment of staphylococcal nuclease, containing residues 114-145. Residue 140, which is normally tryptophan in the native protein, was replaced with phenylalanine during the synthesis since this helps eliminate side reactions during assembly of the chain and since the tryptophan residue is known to be unessential to activity. The final product was removed from the solid support and is at present under purification by ion exchange chromatography and affinity chromatography. The latter procedure is applicable because the fragment under consideration complements with residues 1-126 of nuclease to form a noncovalently stabilized complex with enzymic activity. Thus, 1-126, immobilized on Sepharose, may be used to catch the "good" synthetic material.

Significance to Biomedical Research: Former methods of solid-phase synthesis required at least four days for the synthesis of bradykinin while the present technique permits completion in about 5 hours. If this method can be extended to other peptides, such as the 32 residue peptide discussed above, it should enable investigators to study many more problems by this method than could previously have been studied in a comparable time.

Proposed Course of Project: Further modifications of the protecting groups employed and the methods for purification of products will be developed.

Publications:

Corley, L., Sachs, D.H., and Anfinsen, C.B.: Rapid solid-phase synthesis of bradykinin. Biochem. Biophys. Res. Comm. 47: 1353-1359, 1972.

Serial No. NIAMDD-LCB-11
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Isolation, structural determination, and synthesis of human interferon

Previous Serial Number: None

Principal Investigators: Christian B. Anfinsen
Lila Corley
Robert F. Goldberger

Man Years:

Total: 1.5
Professional: 0.5

Project Description:

Objectives: The availability of fairly large amounts of crude human interferon from NIAID, through a commercial contract involving the use of human leukocyte cell cultures challenged with Newcastle virus, makes it feasible to consider the isolation of pure agent for subsequent structural and biological study. Eventual synthesis of human interferon would appear to be the only practical approach to the use of the substance clinically since it is present in such minute amounts in biological sources.

Methods Employed and Major Findings: The crude culture fluid has been exposed to a variety of pH and denaturing conditions and to gel filtration, ion exchange chromatography, affinity chromatography on lecten columns, and electro-focusing. Human interferon is stable for long period to 0.1 M acetic acid, pH 3, at room temperature and has been purified extensively by three passes of dialyzed, lyophilized crude material through G100 Sephadex. The final material has been examined by disc gel and electro-focusing and is contained in a glycoprotein fraction of molecular weight approximately 25,000. Although it is known to be a glycoprotein, its terminal sialic acid has apparently been completely removed by the extended solution of pH 3. The best material obtained so far has been injected into goats with the hopes of producing an antibody that might be used for solid phase immunoabsorption. Such a step would eliminate the tedious and wasteful early procedures used to date.

Significance to Biomedical Research: As mentioned above, knowledge about this broad spectrum antiviral substance will hopefully permit the organic synthesis of the molecule, or of the active part of its structure, for clinical testing.

Serial No. NIAMDD-LCB-12
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Isolation and characterization of trichocysts from *Paramecium aurelia*.

Previous Serial Number: NIAMDD-LCB-13

Principal Investigator: Edward Steers, Jr.

Other Investigator: Sidney Pollack

Man Years:

Total: 1.0
Professional: 1.0

Project Description:

Objectives: The trichocyst of Paramecium is an organelle whose morphological properties are virtually identical to those of skeletal muscle. While the apparent homology of structure is clear, the functional aspects of these two organelles seems quire different. The objectives of the current study include the isolation and characterization of the structural proteins found in the wild-type cell and subsequent comparison with those found in various mutant strains which have now been identified and isolated. Certain of these mutants represent developmental variants whose study can give some understanding of the developmental processes involved in the formation of what appears to be a prototype muscle found in this protozoan.

Methods Employed: Paramecia were grown in five gallon carboys in a culture media of grass extract (Steers, E., Jr., PNAS 48: 867, 1962). Trichocysts were obtained by differential centrifugation of partially concentrated animals and were either thermally solubilized or solubilized by incubating in the denaturants sodium dodecyl sulfate or guanidine hydrochloride. Polyacrylamide gel electrophoresis and gel filtration chromatography were performed on both the heat solubilized trichocysts and on trichocysts solubilized in either SDS or GHCl.

Major Findings: Trichocysts have been solubilized in aqueous solution at neutral pH by heating to temperatures of 70° for 10 minutes. The product of such solubilization appears identical to that achieved by the previous method using trichocysts solubilized in sodium dodecyl sulfate (SDS) when examined by SDS polyacrylamide disc gel electrophoresis. While the solubilization is virtually total in distilled water, no more than 20% of the total protein solubilizes in the presence of salts even in amounts as low as 0.05 M. Heat solubilized trichocysts show two principal components when run on standard 10% polyacrylamide disc gels and two bands when run on SDS disc gels. The relationship between the two bands seen on standard gels and the two bands seen on SDS gels is unknown at this time. The two bands seen on SDS disc gels have been separated from one another by gel filtration on Sephadex G-200 in buffers containing 0.1% SDS or 5.0 M GHCl. The peaks separated by this method have been partially characterized chemically. Although very similar in their overall amino acid composition significant differences exist between the two components in certain residues. The two components differ in molecular weight representing monomer-dimer and have been tentatively characterized as representing two polypeptide chains designated A and A'. The ability to solubilize the material in aqueous solution by heat now allows certain chemical studies which were precluded by the use of SDS and GHCl in previous work.

Significance to Biomedical Research: This work represents a continuation of our objectives to characterize structural proteins related to the cell surface properties using specialized systems in microorganisms.

Proposed Course of Project: Following characterization of the wild-type and mutant structural proteins we hope to obtain some information on the types of genetic lesions found in the mutants and thus determine how this organelle develops and relates to contractile proteins associated with the cell surface.

Publications:

Pollack, S. and Steers, E., Jr., Exp. Cell Res., 78: 186, 1973.

Serial No. NIAMDD-LCB-13
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Isolation and characterization of a unique invertebrate hemoglobin from Paramecium.

Previous Serial Number: None

Principal Investigator: Edward Steers, Jr.

Man Years:

Total: 0.5
Professional: 0.5

Project Description:

Objectives:
1) To purify the heme containing pigment found in the cytoplasm of the protozoan, Paramecium.
2) To chemically characterize this invertebrate hemoglobin and determine its relationship to those vertebrate hemoglobins already characterized.

Methods: Paramecia are grown as previously dexcribed in five gallon carboys on grass extract and Aerobacter cloacae (Steers, E., Jr., PNAS 48: 867, 1962). The cells are harvested by passage through a commercial cream separator. Final concentration is achieved by centrifugation in a clinical centrifuge. The packed cells are resuspended in approximately two volumes of Tris-HCl buffer, pH 7.5 which is adjusted to 0.2 M with raffinose to prevent the swelling and lysis of mitochondria. The suspension of cells is then homogenized in a Dounce tissue homogenizer at 0°. The lysed cells are then centrifuged retaining the supernatent solution. This solution, which appears pink in color is further fractionated with ammonium sulfate retaining the 50 to 65% fraction. Following dialysis the sample is chromatographed on columns of Sephadex G-75 which is monitored spectrophotometrically at 416 nm. The appropriate fractions are pooled and concentrated by precipitating with ammonium sulfate at 65% saturation.

Major Findings: The fraction isolated by the procedures outlined above appears to be approximately 70% pure and represents on the order of 40μg of hemoglobin per ml of packed cells. The heme protein has an adsorption spectrum characteristic for the reported mammalian hemoglobin and myoglobin pigments and most closely resembles sperm whale myoglobin. The initial experiments clearly show that this pigment is not one of the cytochromes, and differs significantly from the hemoglobin and myoglobin so far characterized in that the heme appears to be covalently bound to the globin. This situation appears to be unique for any hemoglobin or myoglobin reported to date.

Of additional interest is the physiologic role of this molecule in Paramecium. It would appear neither to function as a transport substance for oxygen as oxygen freely diffuses through the cell nor does it appear to serve as a storage mechanism being in too small a quantity to serve the normal metabolic needs of the organism under anaerobic conditions.

Significance to Biomedical Research: Many invertebrate hemoglobins have been identified in a variety of sources. This is the first instance of a molecule of this class appearing in the protozoa. Of especial interest is the possible role of this molecule which may be quite different than the role ascribed to these pigments in higher organisms as well as the apparent structural difference where the heme group is covalently bound to the protein. While the spectral and functional characteristics of the molecule are clearly those of the hemoblobin class of compounds, the covalent attachment of the heme is characteristic of the cytochromes.

Proposed Course of Project: Following the successful purification of Paramecium hemoglobin, a full characterization is anticipated including sequence analysis.

Serial No. NIAMDD-LCB-14
1. Lab. Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The nature of the effect of alterations in the first structural gene of the histidine operon on repression of the operon.

Previous Serial Number: NIAMD-LCB-17

Principal Investigator: Marilyn Meyers

Other Investigators: Robert F. Goldberger
Antonio Ballesteros
Marco Soria

Cooperating Unit: Columbia University, College of Physicians and Surgeons (John S. Kovach)

Man Years:

Total: 0.5
Professional: 0.2
Other: 0.3

Project Description:

Objectives: On the basis of previous studies on the kinetics of repression of the histidine operon in Salmonella typhimurium, we suggested that the first enzyme for histidine biosynthesis (G enzyme) plays a previously unrecognized role in control of the operon. Subsequent studies showed that organisms producing a feedback resistant G enzyme could not be repressed by triazolalanine (TRA), an analogue of histidine which does repress organisms producing wild type G enzyme. We wanted to determine whether this effect of the G enzyme on repression is cis or trans, dominant or recessive.

Methods Employed: An episome bearing a wild type G gene was introduced into a feedback resistant strain. The merodiploid was tested for its ability to be repressed by TRA. Similarly, an episome bearing a mutation in the G gene which renders the G enzyme feedback resistant was introduced into a strain with a normal G gene, and this merodiploid was tested for its ability to be repressed by TRA.

Major Findings: Whereas the feedback resistant strain itself was not repressible by TRA, the merodiploid of this strain containing a wild-type eqisome was repressible by TRA. Whereas the feedback resistant episome itself (in a deletion strain) was not repressible by TRA, this episome in the strain containing a wild-type G gene was repressible by TRA. These findings demonstrate that the effect of the normal G enzyme acts in a *trans* dominant fashion. We conclude that the enzyme exerts its effect on the repression process not by acting locally at its site of synthesis but by acting as a freely diffusible gene product, as expected for a regulatory molecule such as a repressor.

Significance to Biomedical Research: The fact that the first enzyme of a biosynthetic pathway has a regulatory effect on its own biosynthesis and on the synthesis of those enzymes encoded in the same operon may be important to the elucidation of the mechanisms controlling biosynthetic pathways.

Proposed Course of Project: Terminated.

Publication:

Kovach, J.S., Ballesteros, A.O., Meyers, M., Soria, M. and Goldberger, R.F.: A *cis*/*trans* test of the effect of the first enzyme for histidine biosynthesis on regulation of the histidine operon. *J. Bacteriol*. 114, 351-356, 1973.

Serial No. NIAMDD-LCB-15
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mutations in the first structural gene of the histidine operon which alter regulation of the operon.

Previous Serial Number: NIAMD-LCB-16

Principal Investigator: Marilyn Meyers

Cooperating Unit: Department of Biological Sciences, Purdue University
(Mark Levinthal)

Man Years:

Total: 1.6
Professional: 0.8
Other: 0.8

Project Description:

Objectives: On the basis of previous work done on the kinetics of repression of the histidine operon in Salmonella typhimurium, we suggested that the first enzyme for histidine biosynthesis plays a previously unrecognized role in control of the histidine operon. A prediction of this hypothesis is that certain mutations in the first structural gene (*G* gene) of the operon would lead to alterations in the structure of the first enzyme which affect regulation of the histidine operon. Although a number of mutations affecting the catalytic function of this enzyme are known, none of these affect the process of repression. We have been attempting to find mutations in the first structural gene which do alter the repression process.

Methods Employed: Several selection procedures have been used. As reported previously, initial attempts to obtain mutations in the *G* gene involved selecting histidine auxotrophs from a strain containing two normally functioning genes for each of the histidine biosynthetic enzymes except for the *G* gene which is represented only once. Theoretically, all histidine auxotrophs derived from this strain should have mutations in the *G* gene. Although this method has led to the isolation of several strains bearing mutations in the *G* gene, this has been an extremely rare event, and none of these auxotrophs shows any change in the process of repression.

To be certain that every organism selected for study contains a new mutation in the *G* gene another procedure was tried. A large number of prototrophic revertants of several different complete *G* mutants were examined for alterations in regulation of the histidine operon. None was found to have a regulatory defect.

A third approach has been the selection of feedback resistant mutants, mutants in which the G enzyme is catalytically active but insensitive to inhibition by histidine. We had previously demonstrated that such mutants are altered in their ability to be repressed. To further study this phenomenon, we developed a procedure which allowed the direct selection of feedback resistant mutation in the G gene of episomes carrying the histidine operon. Twenty feedback resistant episomes were isolated. Several were found to produce a G enzyme highly resistant to inhibition by histidine. Using these episomes to make merodiploid organisms, we were able to demonstrate that the effect of the G enzyme on repression of the histidine operon was trans dominant (see Project Report #14). An exciting finding of these studies was that one of the feedback resistant episomes is not under repression control. This strain may be the first representative of the class of constitutive G gene mutations predicted by our hypothesis.

Major Findings: An episome bearing the histidine operon containing a mutation to feedback resistance has been isolated. The strain carrying this episome is not under repression control. This finding supports the hypothesis that the G enzyme plays a direct role in repression of the histidine operon.

Significance to Biomedical Research: If alterations in the first enzyme for histidine biosynthesis which affect repression of the histidine operon are found, they will constitute proof of our hypothesis, that a biosynthetic enzyme may have regulatory properties which allow it to control its own synthesis as well as the synthesis of the other biosynthetic enzymes.

Proposed Course of Project: Terminate after publishing results.

Serial No. NIAMDD-LCB-16
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The effect of transfer RNA on the activity of G enzyme, the first enzyme in the histidine pathway.

Previous Serial Number: None

Principal Investigators: Tikva Vogel

Other Investigators: Henry Kronenberg
Robert F. Goldberger

Man Years:

Total: 0.5
Professional: 0.5

Project Description:

Objectives: Histidine inhibits the G enzyme, the first enzyme in the histidine biosynthetic pathway in Salmonella typhimurium. Charged $tRNA^{His}$ plays a role in repressing the histidine operon. G enzyme may play a role in gene regulation as well, and is known to bind specifically charged $tRNA^{His}$. We want to see if $tRNA^{His}$ has any specific effect on the activity of G enzyme itself.

Methods Employed: A sensitive new assay for G enzyme was developed because nucleotides interfere with the standard spectrophotometric method. The G enzyme catalyzes the reaction

$$PRPP + ATP \qquad PRATP + PPi.$$

The assay uses the fact that C^{14} PRPP does not stick to charcoal at acid pH, but C^{14} PR-ATP does stick. Purified tRNA species were obtained by reverse phase chromatography and charged using standard techniques.

Major Findings: At very low concentration tRNA partially inhibits G enzyme. There was no inhibition of G enzyme from a mutant with altered G enzyme feedback sensitivity to histidine. Preliminary studies show no specificity for charged or uncharged $tRNA^{His}$. ATP and PRPP reverse both the binding and the inhibition by tRNA. Studies have been limited by the scarcity of charged $tRNA^{His}$.

Significance to Biomedical Research: G enzyme--as the first enzyme in the histidine operon and pathway--may take part in histidine regulation on both the genetic and enzymatic level. Interaction with tRNA may shed light on the growing field of protein-nucleic acid interactions.

Proposed Course of Project: With newly available charged $tRNA^{His}$, the specificity and inhibition kinetics of the reaction will be determined, using normal and mutant *G* enzymes.

Serial No. NIAMDD-LCB-17
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: In vitro transcription of the histidine operon.

Previous Serial Number: None

Principal Investigators: Robert Goldberger
Roger Deeley
Marilyn Meyers
Francesco Blasi

Man Years:

Total: 1
Professional: 0.5
Other: 0.5

Project Description:

Objectives: An *in vitro* system was developed for transcription of the histidine operon of *E. coli* carried in the genome of a defective 080 transducing phage in order to study regulation of expression of the operon in a more simplified system than that provided by the intact bacterial cell *in vivo*.

Methods Employed: DNA was prepared from 080 and 080d*his* and the strands of these DNA preparations were separated by standard techniques. *In vitro* transcription was carried out by the method described by de Crombrugghe, with slight modifications. Tritiated UTP was included in the incubation mixtures so that the RNA synthesized *in vitro* would be radioactively labelled. RNA-DNA hybridization techniques were used to determine complementarity of the RNA made *in vitro* to the individual strands of 080 and 080d*his*. Sucrose density gradient centrifugation was utilized as a means for determining the size distribution of the RNA species synthetized *in vitro* from the phage DNA template.

Major Findings: Messenger RNA of the histidine operon was synthetized *in vitro*. This RNA was detected by hybridization to single strands of both 080 and 080d*his* DNA, and by competition of this hybridization with unlabelled histidine messenger RNA which had been synthesized *in vivo*. Under the conditions employed, histidine operon RNA amounted to about 15% of the total RNA which was synthesized *in vitro* from the 080d*his* DNA template. The RNA complementary to the histidine operon was synthesized on the "sense" strand (the R strand) in the form of a polycistronic message with a sedimentation coefficient (about 38S) very close to that observed for the histidine messenger RNA synthesized *in vivo*. Synthesis of the histidine operon RNA appears to be subject to control *in vitro*.

Significance to Biomedical Research: The development of an *in vitro* system for studying regulation of a biosynthetic system will provide the means for determining the mechanism of such regulation. This mechanism has bearing on many problems of interest in the biomedical sciences.

Proposed Course of Project: The *in vitro* system will be used to determine the effects of histidine transfer ribonucleic acid, the first enzyme for histidine biosynthesis, and other molecules known, or suspected, to play a role in regulating expression of the histidine operon.

Serial No. NIAMDD-LCB-18
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Purification of the first enzyme of the histidine operon by affinity chromatography

Previous Serial Number: None

Principal Investigators: Loren G. Lipson
Robert F. Goldberger

Man Years:

Total: 0.5
Professional: 0.5

Project Description:

Objectives: The principal objective was to make use of purified specific antibodies to the first enzyme of the histidine operon (the G enzyme) as a means of purification of the G enzymes from various mutant species of Salmonella which previously had been isolated only with great difficulty.

Methods Employed: Specific antibodies to the G enzyme were produced by repeated sensitization injections of a goat with pure G enzyme. The antibodies isolated one week after the third inoculation were found to have a titer of 5 mg/ml of specific anti-G enzyme activity. These antibodies were purified upon a sepharose column which had been coupled to the monomeric form of the G enzyme. One fraction of these antibodies eluting in guanidine·HCl was adsorbed with an extract of a G deletion mutant and passed through the sepharose-G column again. The fraction eluting in 2 M guanidine·HCl was dialyzed and coupled to sepharose with cyanogen bromide. The resulting column was used for the studies.

Major Findings: It appears that wild type G enzyme will bind to the sepharose-antibody column and can be eluted. Preliminary findings show a 500-fold purification in one step.

Significance to Biomedical Research: The development of a one-step purification for the first enzyme of the histidine operon will allow the rapid purification of the enzyme produced by many mutants for the gene associated with this enzyme. Analysis of differences in these enzymes will aid in the understanding of regulation of the entire histidine operon.

Proposed Course of Project: The purification of various mutants of G enzyme activity will be continued with particular emphasis on the purification of the feedback resistant G enzyme.

Serial No. NIAMDD-LCB-19
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Reports
July 1, 1972 through June 30, 1973

Project Title: Correlation of cross reacting material with enzyme activity and mutant type of the first enzyme of the histidine operon (the G enzyme).

Previous Serial Number: None

Principal Investigators: Loren G. Lipson
Robert F. Goldberger

Man Years:

Total: 0.5
Professional: 0.5

Project Description:

Objectives: The principal aim of this study is to correlate mutation type with both G enzyme activity and the presence of cross reacting material determined by specific antibody to the G enzyme.

Methods Employed: Specific antibodies to pure G enzyme were isolated from serum of previously sensitized goat. These antibodies were adsorbed against the extract of a mutant with a G gene deletion. The antibody preparation was then reacted on double immunodiffusion plates against extracts of various Salmonella mutants in the G gene. The G activity of these crude extracts were also determined, using a spectrophotometric assay.

Major Findings: It appears that derepressed cultures of various G mutants can indeed be screened by these methods.

Significance to Biomedical Research: The possibility of detection of cross reacting material in missense and nonsense mutants promises to add more to the understanding of regulation of the histidine operon.

Proposed Course of Project: The project will involve all G mutants. Any of the nonsense mutants with CRM will be further investigated in regard to protein structure and possible function.

Serial No. NIAMDD-LCB-20
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Interaction between the first enzyme for histidine biosynthesis and His-tRNA.

Previous Serial Number: NIAMD-LCB-15

Principal Investigators: John S. Kovach
Tikva Vogel
Robert F. Goldberger
Roger Deeley
Marilyn Meyers

Man Years:

Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: On the basis of our previous work on the kinetics of derepression of the histidine enzymes we postulated that the first enzyme for histidine biosynthesis plays a previously unrecognized role in repression of the histidine system in *Salmonella typhimurium*. Because it has been known for some time that His-tRNA is required for repression of the histidine system, it appeared to us that one test of our hypothesis is to determine whether the first enzyme for histidine biosynthesis interacts with His-tRNA. Using crude tRNA, we previously found that the enzyme had a high affinity for tRNA and that it bound His-tRNA in preference to tRNA which has been aminoacylated with any other amino acid. In the present study, we used various partially purified species of tRNA, to test the question of whether the enzyme binds the aminoacylated form of His-tRNA in preference to the deacylated form.

Methods Employed: Partially purified preparations of various species of tRNA were obtained from Dr. Donald Kelmers of the Oak Ridge National Laboratory. In addition, tRNA^{His} was purified to homogeneity from *Salmonella typhimurium* (both wild type and *T*-mutant) by the published procedure. The *G* enzyme and histidyl-tRNA synthetase were purified by the published procedures. Binding of the *G* enzyme to tRNA was studied by using the nitrocellulose filter binding technique of Yarus and Berg.

Major Findings: We found that for species of tRNA other than tRNA^{His}, the *G* enzyme does not distinguish between the aminoacylated and deacylated forms. In contrast, the G enzyme binds aminoacylated histidyl-tRNA in preference to deacylated $tRNA^{His}$. Thus, the enzyme not only binds the histidine species of tRNA in preference to all other species, but also binds the aminoacylated form of histidine tRNA preferentially. The possibility that histidyl-tRNA carries out its regulatory role as a complex with the G enzyme is consistent with these findings. Binding studies with His-tRNA from the T mutant are still in progress.

Significance to Biomedical Research: The finding that His-tRNA, a macromolecule known to be required for repression of the histidine system, binds with a very high affinity to the first enzyme of the histidine biosynthetic pathway, may be an important clue to understanding the mechanisms which regulate biosynthetic metabolic pathways.

Proposed Course of Project: We are studying the relative affinities of the G enzyme to wild type His-tRNA and T-mutant His-tRNA to determine whether the regulatory defect in T mutants involves a defect in the ability of the altered His-tRNA to bind to the G enzyme.

Publications:

Vogel, T., Meyers, M., Kovach, J.S. and Goldberger, R.F.: Specificity of interaction between the first enzyme for histidine biosynthesis and aminoacylated histidine transfer ribonucleic acid. J. Bacteriol. 112, 126-130, 1972.

Goldberger, R.F. and Kovach, J.S.: Regulation of histidine biosynthesis in *Salmonella typhimurium*. In Horecker, B.L. and Stadtman, E.R. (eds.): *Current Topics in Cellular Regulation*, vol. 5, New York, Academic Press.

Serial No. NIAMDD-LCB-21
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Interaction between the first enzyme for histidine biosynthesis and Ø80dhis DNA.

Previous Serial Number: NIAMD-LCB-18

Principal Investigators: Tikva Vogel
Robert F. Goldberger
Roger Deeley

Other Investigators: Mark Levinthal
Carmello Bruni
Francesco Blasi

Man Years:

Total: 1.5
Professional: 1.0
Other: 0.5

Project Description:

Objectives: We previously proposed that the first enzyme for histidine biosynthesis, the G enzyme, plays a role in regulation of the histidine operon. If this enzyme acts at the genetic level, it would be expected to interact with some regulatory element in the DNA of the histidine operon. The recent isolation of a phage carrying the histidine operon in place of some of its own genes has made it possible to test this possibility.

Methods Employed: Radioactively labelled and unlabelled DNA was isolated from Ø80 and from Ø80dhis. Interaction between these preparations of DNA and the G enzyme was studied by the filter binding assay used by Riggs and Bourgois to study binding of the lac repressor to the lactose operon. Competition between cold Ø80dhis DNA and radioactive Ø80dhis DNA and between cold Ø80 DNA and radioactive Ø80dhis DNA was studied to determine whether there is any specific interaction between the enzyme and the bacterial genes carried in the phage genome.

Major Findings: The enzyme binds Ø80dhis DNA. As expected, this binding is completely inhibited by unlabelled Ø80dhis DNA. However, unlabelled Ø80 DNA was not able to inhibit completely. Approximately 30% of the binding to Ø80dhis DNA is maintained even in the presence of a two thousand-fold excess of unlabelled Ø80 DNA. Thus, the enzyme binds specifically to the bacterial DNA carried in the genome of Ø80dhis. We suggest that it binds specifically to some regulatory element in the histidine operon.

Significance to Biomedical Research: The finding that an enzyme may serve as a regulatory protein controlling the rate of its own biosynthesis may be of general importance in repression control.

Proposed Course of Project: We will study what effect on the binding of *G* enzyme to Ø80d*his* DNA is obtained by the addition of aminoacylated histidine tRNA and deacylated tRNA^{His}. We will also study whether the enzyme binds to the DNA of a Ø80d*his* in which there is an operator mutation in the histidine operon.

Serial No. NIAMDD-LCB-22
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Regulation of enzyme synthesis in mammalian cells in tissue culture.

Previous Serial Number: NIAMD-LCB-19

Principal Investigator: Loren G. Lipson

Other Investigator: Henry Kronenberg

Man Years:

Total: 1.5
Professional: 1.5

Project Description:

Objectives: To study repression of amino acid biosynthetic pathways in mammalian cells in tissue culture.

Methods: In order to achieve the above objective, a tissue culture laboratory was constructed and equipped. The laboratory was set up so that tissues could be cultured either in culture dishes or in suspension. Six different mammalian liver cell lines were then screened for presence of several amino acid biosynthetic pathways using modified assay systems. In addition several cell lines were adapted to growth in suspension culture in order to increase the cellular yield for enzyme isolations. Assays were developed to study the pathway for serine biosynthesis. Cells were grown in spinner culture under conditions in which growth was limited by the amount of serine available in the medium, and the intracellular concentrations of the serine biosynthetic enzymes in these cells were compared with the concentrations in cells grown on excess serine.

Major Findings: Although these experiments were performed in several different ways, limiting serine availability to various degrees, and although several different cell lines were used, no evidence was obtained for repression or induction of the serine biosynthetic enzymes.

Proposed Course of Project: Terminated.

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL PHARMACOLOGY

I. PROTEIN STRUCTURE AND MECHANISM OF ENZYME AND HORMONE ACTION

Tryptophan Synthetase of *Escherichia coli*

The B protein of tryptophan synthetase contains pyridoxal-P as a cofactor and catalyzes a number of pyridoxal-P dependent reactions. We are studying the structure and function of this enzyme and are determining how the B protein is regulated by the presence of the A protein in the tryptophan synthetase AB complex.

The B protein has been purified and has been crystallized for the first time in a new purification procedure which gives a high yield of homogeneous enzyme. The catalytic properties of the new preparation have been compared with those of B protein purified by the standard procedure of Wilson and Crawford. The results indicate that the standard purification procedure, which contains 2 heat steps at 56° and 80°, results in some alteration of the properties of the native B protein and that this alteration is avoided in the new, improved preparation.

(Drs. O. Adachi and E. W. Miles)

The mechanism of the conversion of L-serine to pyruvate by the B protein is being investigated. Studies with α-D-serine show that the overall reaction is rate-limited by the removal of the α-proton of L-serine. They have also identified the enzyme-substrate intermediate which accumulates before this step. The finding that the rate of the overall reaction is dependent on an ionizing group with a pK of 6.7 suggests that a histidyl residue may serve as a base-catalyst in the removal of the α-proton. Further evidence for the role of an essential histidyl residue in the B protein has been provided by chemical modification studies with diethylpyrocarbonate and by photooxidation studies of the B protein in the presence of L-serine and pyridoxal-P.

(Dr. E. W. Miles)

Histidine Ammonia-Lyase

Histidine ammonia-lyase catalyzes the α,β elimination of NH_3 from histidine to produce urocanic acid. We have been trying to elucidate the mechanism of this catalysis as well as to characterize the nature of the participating groups in the protein.

Mechanism of Action. Steady state and transient state kinetic studies have been carried out using L-histidine and its analog 4-fluoro-L-histidine. With the substrate analog the rate determining step of the reaction precedes the dissociation of the amino enzyme intermediate which is the postulated rate determining step in the case of L-histidine. This substrate analog may therefore supply important clues for the mechanism of bond breakage in the elimination reaction.

(Drs. C. B. Klee, L. A. Cohen [A:LC], K. L. Kirk [A:LC], and P. McPhie [A:LMB])

Nuclear magnetic resonance studies of the effect of the substrate 4-fluoro-L-histidine on the interaction of the enzyme - Cd^{2+} complex with Cl^- ions are being carried out in order to elucidate the role of metal ions in the reaction catalyzed by the enzyme.

(Drs. H. J. C. Yeh [A:LC], C. B. Klee, L. A. Cohen [A:LC], and K. L. Kirk [A:LC])

Active Site Studies. The stereospecific, irreversible inhibition of the enzyme by L-cysteine has been characterized further. L-Cysteine interacts with a residue at the active site of the enzyme which, upon reduction with $[^3H_2]NaBH_4$ yields ^3H alanine (R. B. Wickner) or upon treatment with Na sulfate yields cysteic acid. The reaction of the enzyme with cysteine occurs in two steps: (1) a fast, irreversible inactivation and incorporation of cysteine followed by (2) a slow modification resulting in UV absorption changes. A peptide has been isolated from a pronase digest of the inactivated enzyme which contains the radioactive cysteine and has the UV absorption peak at 340 nm characteristic of the inactivated enzyme.

(Dr. C. B. Klee)

Lactose Synthetase

Studies of the guanidination of α-lactalbumin have shown that the ε amino groups of this protein may be completely converted to guanidino groups without affecting the activity of α-lactalbumin in the lactose synthetase complex. This reaction is shown to be a useful way of introducing a stable radioactive label into the protein for binding studies.

(Drs. K. S. Iyer [M:LGCB], W. A. Klee [M:LGCB], and C. B. Klee)

Glycyl tRNA Synthetase

The general importance of the previously reported susceptibility of this enzyme to inhibition by inorganic sulfide is now indicated. Six additional amino acyl tRNA synthetases from yeast and one from rat liver have been shown to be similarly susceptible, and the property is evident in fresh extracts which have had very little possibility of being degraded or in some way changed prior to the test. A regulatory process involving the opening and closing of an enzyme's disulfide bond is indicated, which should work in conjunction with a regulating factor that has been under study for several years.

(Dr. S. Black and Mrs. B. Hazel)

Relationships of Thyrotropin to Exophthalmogenic Factor and Studies of Thyrotropin Action on the Thyroid Gland and Retro-orbital Tissues

Previous work demonstrated that TSH was exophthalmogenic but that partial pepsin digestion of TSH could yield an exophthalmogenic fragment of the TSH molecule which had lost its thyroid stimulating ability. This 20,000 molecular weight fragment consists of all of the β subunit and an amino terminal fragment of the α subunit of TSH. This fragment and TSH can cause exophthalmos in fish, guinea pigs, and mice.

Thyrotropin-induced exophthalmos in the guinea pig is associated with a two-fold increase in the glycosaminoglycan content of the predominant tissue of the retro-orbital space, the Harderian gland. Hyaluronic acid is the major glycosaminoglycan of the normal gland and accounts for over 90% of the two-fold increase. Sulfated glycosaminoglycans account for the remainder. Since the increase in sulfated glycosaminoglycans is proportional to the increase in hyaluronic acid, nondialyzable [^{35}S]sulfate incorporation into the Harderian gland is a sensitive index of these glycosaminoglycan increases. Dialyzable [^{35}S]sulfate incorporation measures an early effect of thyrotropin administration, *i.e.*, the conversion of inorganic sulfate to an activated sulfate derivative, phosphoadenosinephosphosulfate.

The glycosaminoglycan increases in exophthalmos can be induced by crude thyrotropin preparations, by purified and homogeneous preparations of thyrotropin, or by the 20,000 molecular weight proteolytic derivative of the thyrotropin molecule which has effectively no thyroid stimulating activity but is still exophthalmogenic in fish bioassays. These findings again indicate that thyrotropin is exophthalmogenic but that the determinant responsible for the exophthalmogenic activity is not the same as that responsible for thyroid stimulation. They suggest that the absence of thyroid stimulating activity in the serum of patients with exophthalmos does not exclude a role for thyrotropin in the pathogenesis of this disease.

Plasma membranes have been prepared from bovine thyroid cells and have been characterized as being reasonably free of other subcellular structures by electron microscopy and enzyme marker analysis. Bovine thyrotropin specifically binds to these membranes, and this binding can be correlated with adenylate cyclase activation. The binding of thyrotropin to the membranes is immediate, is inversely dependent on temperature, is inhibited by increasing salt concentrations, and is not affected by a range of mono-, di-, and trisaccharides. The thyroid-stimulating activity of the thyrotropin preparations is unaffected by incubation with membranes under conditions of the binding assay.

Both the α and the β subunits of bovine thyrotropin bind to thyroid membranes, β better than α on a molar basis; only the β subunit, however, inhibits thyrotropin binding at the concentrations tested. Mouse tumor thyrotropin is a potent inhibitor of bovine thyrotropin binding; human thyrotropin is not as effective. Since γ-globulin preparations with or without long acting thyroid-stimulating activity inhibit thyrotropin binding only slightly at high γ-globulin concentrations, the long acting thyroid stimulator itself appears to have no effect on thyrotropin binding to thyroid membranes. The TSH receptors of thyroid and retro-orbital tissues can thus be functionally distinguished, and additional credence is given to the possibility that alterations of normal hormone-receptor interactions can result in human disease.

Since one of the two subunits of luteinizing hormone (LH) has a primary amino acid sequence that is very nearly identical to the α subunit of TSH, we have examined the effect of LH on TSH binding and have tried to correlate this effect with several parameters of biological activity: adenylate cyclase activity of beef thyroid plasma membranes and the oxidation of [1-^{14}C]glucose by dog thyroid slices. These effects were, in turn, compared to those of the α and β subunits of TSH. The results show a high correlation between the binding parameter and these physiological responses; they emphasize the importance of chemical criteria rather than *in vivo* biologic potency as a measure of purity.

An antibody has been made to the exophthalmogenic fragment of the TSH molecule. Its cross reactivity with TSH and the subunits of TSH is under investigation.

(Drs. L. D. Kohn, S. M. Amir, R. L. Tate, J. Wolff [A:CE], and R. J. Winand [University of Liege, Liege, Belgium])

Procollagen and Procollagen Peptidase

Procollagen peptidase from bovine tendons has been purified. The final preparations contain two active components, molecular weights ~ 70,000 and ~ 140,000. Both active species are precipitated by an antibody to the enzyme and by immunoelectrophoretic analysis have common antigenic components. Since rechromatography of any one component always yields both components, the enzyme is presumed to be a polymerizing system composed of 70,000 molecular weight monomers. Both forms work on both pro α1 and pro α2 chains and are EDTA inhibited. The 70,000 molecular weight species is not Ca^{2+} reactivated, whereas the 130,000 molecular weight species is. They prefer native procollagen (K_m 10^{-6} M) as substrate as opposed to the individual pro α1 and pro α2 chains (K_m 10^{-3} M). The enzyme is an endopeptidase which cleaves the amino terminal peptide en bloc. The amino acid composition and molecular weight of the excised peptide have been determined.

Procollagen synthesis has been studied in L929 cells, a fibroblast culture of a mouse tumor. These cells are analogous to dermatosparaxic in their low level of procollagen peptidase activity in the media, and approximately 25% of their ribosomal product is procollagen. The cells contain precursor chain aggregates which are disulfide bonded, *i.e.*, the procollagen molecule forms intracellularly and appears to be disulfide bond stabilized.

Three patients with a variant of the Ehlers-Danlos syndrome, a generalized disorder of connective tissue, have detectable amounts of procollagen in extracts of their skin and tendon. The activity of procollagen peptidase, the enzyme which converts procollagen to collagen, was reduced in their skin fibroblast cultures. The clinical manifestations of this syndrome may be related to impaired enzymatic conversion of procollagen to collagen. Skin fibroblast cultures from these patients have an increased synthetic rate of collagenous (collagen and procollagen) protein, possibly related to the inability to convert procollagen to collagen.

(Drs. L. D. Kohn, C. Isersky [A:A&R], C. M. Lapiere [Hopital de Baviere, Liege, Belgium], S. S. Kerwar [Roche Institute of Molecular Biology, Nutley, New Jersey], G. R. Martin [D:LB], and E. C. LeRoy [Columbia University College of Physicians and Surgeons, New York City], Mr. G. Lee [American University], and Mr. G. Poy [A:A&R])

Membrane Transport

The membrane-bound D-lactate dehydrogenase of *Escherichia coli* ML 308-225 has been solubilized and purified to homogeneity. The enzyme has a molecular weight of 75,000 ± 7% and contains approximately 1 mole of flavin adenine dinucleotide per mole of enzyme. Its pH optimum before exposure to Triton X-100 is 7.5 to 8.0; after exposure it is 8.0 to 9.5. The purified enzyme has a K_m value of 1.4×10^{-3} M for D-lactate and 3.0×10^{-2} M for L-lactate before exposure to Triton X-100; after exposure its K_m values are 0.5×10^{-3} M and 2.1×10^{-2} M for the same substrates, respectively. The enzyme is not active with D-glycerate, L-glycerate, succinate, malate, D-tartrate, L-tartrate, mesotartrate, n-propanol, or isopropanol as substrates and oxidized diphosphopyridine nucleotide has no effect on the catalytic conversion of D-lactate to pyruvate. The enzyme is not inhibited by p-chloromercuribenzoate, N-ethylmaleimide, cr 5,5'-dithiobis(2-nitrobenzoic acid), but is inactivated by treatment with 2-hydroxy-3-butynoic acid.

Recent experiments demonstrate that guanidine extracts from wild type vesicles containing D-lactate dehydrogenase activity are able to reconstitute D-lactate-dependent oxygen consumption and active transport in membrane vesicles from an *E. coli* mutant deficient in D-lactate dehydrogenase.

(Drs. L. D. Kohn and H. R. Kaback [Roche Institute of Molecular Biology, Nutley, New Jersey])

Membrane Structure

The membrane-penetrating MN-glycoprotein of the human erythrocyte has been examined by chemical and physical techniques. The intramembranous portion of this glycoprotein is shown to belong to an unusually hydrophobic class of peptides which includes the membrane spanning antibiotic, gramicidin A. Circular dichroism data show that this intramembranous region forms an unusually stable helix in hydrophobic environments, and in the absence of strong perturbations should be helical *in situ*. The length of this helix (35 Å) is of the order of the thickness of the hydrophobic core of the membrane. The intramembranous peptide has a strong affinity for cholesterol and phospholipids and contains covalently bound phosphorous which is not associated with phospholipid.

(Drs. J. P. Segrest and L. D. Kohn)

Structure-Function Studies of Hydroxypyruvate Reductases

The hydroxypyruvate reductase induced in *P. acidovorans* by growth on glyoxylate has a molecular weight of approximately 80,000. From sodium dodecylsulfate gels and ultracentrifuge studies a subunit molecular weight of 40,000 has been calculated for the induced enzyme. The subunits of the induced enzyme are identical and can reassociate to yield a protein having the same physical and kinetic properties as native enzyme. The enzyme has four half-cystine residues, all of which act as free sulfhydryl residues and are important to activity. A single peptide containing two sulfhydryl residues has been isolated from peptide maps of the digested subunit preparations. Substrate protection studies suggest the sulfhydryl residues are at the enzyme active site.

The enzyme binds two moles of DPNH per mole of native enzyme. It has an ordered reaction mechanism in which DPNH binds before the carbonyl substrate.

(Drs. L. D. Kohn and J. M. Utting [Georgetown University])

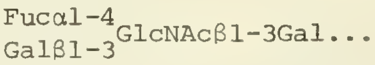
Bacterial Chemotaxis

Studies have been initiated to determine force-response relationships pertaining to chemotactic motions of *E. coli* K_{12} bacteria. Assay techniques for bacterial motion utilize newly developed methods of laser light intensity correlation spectroscopy. The isolation of chemotactic receptors is also being attempted, with subsequent analysis of receptor function when embedded in reconstituted lipid bilayer membranes.

(Drs. R. J. Nossal [CR:PSL] and L. D. Kohn)

II. COMPLEX CARBOHYDRATES

Free oligosaccharides are not antigenic themselves and first have to be coupled to carriers. Published procedures for effecting this coupling are either too harsh for milk oligosaccharides with labile linkages or have a yield of product too low for use with the rarer sugars. For this reason we have investigated several methods for coupling the pentasaccharide, lacto-*N*-fucopentaose II (LNF-II) and have tested the coupled products for antigenicity in rabbits. LNF-II contains the nonreducing terminus:



$$\begin{matrix}\text{Fuc}\alpha 1\text{-}4\\ \text{Gal}\beta 1\text{-}3\end{matrix}\text{GlcNAc}\beta 1\text{-}3\text{Gal}\ldots$$

Two of the methods tested were quite successful: LNF-II was oxidized with I_2 in 95% yield to a gluconic acid derivative. The oxidized LNF-II was then coupled with carbodiimide in 80% yield to Cellex PAB. The oxidized LNF-II was also coupled in high yield to bovine serum albumin using the mixed anhydride technique of Ashwell. Although in the latter technique only 20% of the product is soluble while the remainder precipitates, both the soluble and insoluble material proved to be effective antigens.

Rabbits immunized by standard methods with the Cellex PAB and bovine serum albumin derivatives of LNF-II all developed antibody directed against LNF-II. The antibody was purified by affinity chromatography using LNF-II coupled to "affinose" as absorbant. Antibody was eluted by fucose from the column in high yield and purity.

(Drs. V. Ginsburg, D. A. Zopf, C. A. Mapes, A. M. Jeffrey, and C. Isersky)

III. CELL SURFACES

Structure and Assembly of *Escherichia coli* Membranes

The surface of *E. coli* consists of an inner, or cytoplasmic, membrane, a rigid cell wall, and an outer membrane. All the components of the outer membrane are synthesized elsewhere in the cell: protein on ribosomes within the cell; lipid and lipopolysaccharide (LPS) in the inner membrane. Our goal is to define how these components are organized and assembled during outer membrane biogenesis.

Previous work in this laboratory suggested that newly synthesized LPS molecules are translocated from the inner membrane into discrete specialized regions of the outer membrane. This evidence was obtained by using a mutant of *E. coli* that forms LPS richer in carbohydrate when grown with galactose than when grown in its absence. The outer membrane is denser when it contains more carbohydrate than when it contains less, so fragments of outer membrane derived from cells grown in the presence of galactose can be separated by equilibrium centrifugation from fragments derived from cells grown in its absence. When cells were grown in the absence of galactose, galactose added, and membrane fragments prepared at various times thereafter, within 2-4 minutes (~ 10% of a doubling time), a small percent of the membrane fragments attained the very high density characteristic of fragments from galactose-grown cells. These suggested that they were points where the newly synthesized, dense LPS was inserted into the outer membrane.

These experiments have now been extended to prove that these "insertion points" are truly intermediates in the addition of LPS to the entire outer membrane. When galactose was added to cells for 30 seconds and then removed by dilution, the dense mebrane fragments appeared, and then disappeared, suggesting that dense, new LPS is quickly dispersed throughout the remainder of the membrane. Control experiments indicated that the results did not come from artifacts of mixing or segregation of membrane fragments. Thin sections and negatively stained preparations of these "insertion points" were then examined in the electron miscrope. They consisted of apparently pure single membrane units in spheres of approximately 0.5-1% of the surface area of the original bacterium. From the fraction of membrane that appears in these fragments and the estimated percent yield, it can be calculated that there are 10 to 30 of these insertion points per cell.

Future experiments include large-scale preparations of these membrane fragments so that their composition can be analyzed and compared to that of the remainder of the membrane.

(Drs. C. F. Kulpa, Jr., and L. Leive)

Various mutants have been isolated from the strain used in the above experiment, including auxotrophs for various amino acids, and a strain blocked in the decarboxylation of diaminopimelic acid. These mutants will be used to specifically label protein and the peptidoglycan of cell wall, in order to determine the relationship of newly-synthesized cell wall to "insertion points" of the closely juxtaposed outer membrane, and the method of insertion of protein into the outer membrane.

(Drs. C. F. Kulpa, Jr., and L. Leive)

The lipids of the outer membrane of the above-mentioned strain of *E. coli* have been analyzed and consist of approximately 90% phosphatidylethanolamine, 8% phosphatidylglycerol, and 1% cardiolipin, similar to published values for the outer membrane of *Salmonella*. The method of insertion of lipid into this membrane has been investigated by adding labeled serine to label newly-synthesized phosphatidylethanolamine, while adding galactose to permit synthesis of density-labeled lipopolysaccharide "insertion points." Preliminary experiments indicate that the highest concentration of newly synthesized lipid occurs specifically in those fragments that do *not* serve as insertion points for lipopolysaccharide. These results suggest that there may be separate distinct insertion points for lipid. Subsequent experiments will be necessary to test this hypothesis.

(Drs. A. Kimura and L. Leive)

IV. POLYAMINES

A sensitive and specific method for the automated analysis of polyamines has been further developed and improved in collaboration with Dr. F. Irreverre [A:LBC]. This method is being used to study the amine patterns in bacteria and mammalian cells. We are continuing our studies of enzymes involved in amine biosynthesis and degradation, with emphasis on the propylamine transferase that carries out the synthesis of spermidine in *Escherichia coli*, a new peptidase in *E. coli* that splits glutathionylspermidine to free spermidine, and the spermidine dehydrogenase of *Serratia marcescens* which acts on glutathionylspermidine as well as on free spermidine.

The metabolism of glutathione and other sulfhydryl compounds found in the trichloroacetic acid extracts of *E. coli* is of particular interest because of the conversion of all of the bacterial spermidine and half of the glutathione to a covalent derivative when the bacteria culture approaches the stationary phase. Substantial amounts of this material have been isolated. The structure has been shown to be glutathionylspermidine, in which the glycine carboxyl is linked to spermidine.

(Drs. H. Tabor and C. W. Tabor)

The distribution of a hydroxyputrescine is being studied, as well as its biosynthesis in *Pseudomonas*.

(Drs. H. Fujisawa, H. Tabor, and C. W. Tabor)

V. PROTEIN AND NUCLEIC ACID

Nucleic Acid Metabolism

The DNA polymerase induced by T4 bacteriophage cannot by itself catalyze the stable incorporation of nucleotides using a linear double stranded DNA as the template, although it does catalyze the extensive turnover (incorporation followed by hydrolysis) of residues at the ends of such duplex chains. In contrast, if the DNA unwinding protein specified by the phage gene 32 is present, we find that the T4 polymerase can use a native duplex such as T7 DNA as a template. This "32 protein" had previously been shown by Alberts and his collaborators to be required in stoichiometric amounts for DNA synthesis *in vivo* and to partially unwind DNA and increase the rate of copying of single stranded templates by the T4 polymerase *in vitro*. The product of the copying of native DNA appears not to be covalently attached to the template DNA. When Br dUTP is used as a density label, most of the product bands in alkaline $CsCl_2$ at the same position as "heavy" single strands of DNA, while most of the template bands with "light" single strands. In neutral CsCl the template and product band together at the hybrid density. The size distribution of the template and product are similar as shown by sedimentation in neutral and alkaline sucrose gradients. In the presence of the gene 32 protein, the copying of the single stranded template, poly d(AT), continues until the available triphosphates have been converted into polymer. In the absence of the unwinding protein, synthesis is much slower and does not exceed a single copy of the template.

(Dr. N. G. Nossal)

Factors contributing to the fidelity of DNA replication can be studied with the T4 DNA polymerase, since some temperature sensitive mutations in the structural gene for this enzyme, at permissive temperatures, show either an increased or decreased rate of mutation of markers in other genes. We have purified the polymerases made by a phage with a very high mutation rate (*ts* L88) and a low mutation rate (*ts* CB120) and compared them with the wild type enzyme. Using poly (dA) · (dT) as the template primer, the *ts* L88 mutator enzyme incorporates the noncomplementary nucleotides dGTP and dCTP at a higher frequency than does the wild type enzyme. The misincorporation by the mutant but not the wild type enzyme increases with increasing temperature. The nuclease activity of each enzyme quantitatively removed all noncomplementary residues which were incorporated, so that no difference in the ability of the two enzymes to remove errors could be demonstrated under these conditions.

The polymerase from the antimutator, *ts* CB120, has the same specific activity for nuclease as the wild type enzyme, but only one-sixth the wild type polymerase activity with single stranded T7 DNA as the template. The polymerase and nuclease activities of the mutant have the same thermoability as those of the wild type. A larger proportion of newly incorporated residues are hydrolyzed by the antimutator than the wild type enzyme, in the presence or absence of the 32 protein. While the higher ratio of nuclease to polymerase activity observed with the mutant could result in a greater efficiency in removing erroneous nucleotides, we have not yet ruled out the possibility that the mutant enzyme also makes fewer errors in incorporation.

(Drs. M. S. Hershfield and F. D. Gillin)

Elongation Factors

Evidence obtained earlier in this laboratory has suggested that peptide chain elongation factors may be subject to proteolysis during purification. Accordingly, a method of purification of these factors has been devised incorporating the use of the proteolytic inhibitor, phenylmethylsulfonyl chloride, and avoiding any steps which concentrate the proteins while they are still relatively impure. Using this technique, three polypeptides involved in peptide chain elongation have been isolated from cell-free extracts of *E. coli*; these proteins have molecular weights of 47,500, 35,000, and about 83,000, referred to in this summary as A, B, and C, respectively. In terms of some chromatographic and electrophoretic properties, A, B, and C are analogous to "Tu", "Ts", and "G", respectively. However, A and B have molecular weights that are significantly greater than those reported for Tu (40,000) and Ts (30,000). In rapidly growing bacteria, A, B, and C make up a significant percentage (5-10%) of the nonparticulate protein, and A (47,500) is by far in the largest amount. The bulk of A exists free in the cytoplasm but some is bound noncovalently in a very stable complex to B. There is no evidence that B exists free in the cytoplasm. A, the A-B complex, and C (83,000) have been purified to homogeneity. Either A or A-B is required, along with C, for polypeptide synthesis *in vitro* with highly purified ribosomes; little or no polypeptide synthesis takes place in the absence of C or with C alone. These results suggest that the function provided by B (35,000) is not absolutely required under the conditions of the assay *in vitro*. Experiments are currently underway in this laboratory as well as in collaboration with Dr. C. M. Cashel [CH:LMG] to analyze the requirement and effect of GTP, the novel G tetra and pentaphosphate derivatives of GTP and other phosphorylated compounds on the reactions carried out by the elongation factors.

Experiments are also underway to examine whether there are differences between the A that is complexed with B and the one that is not, by comparing peptide maps prepared from purified A, the A-B complex, and C. The latter comparison might prove to be especially interesting because of the many similar chromatographic and electrophoretic properties between the A-B complex and C.

(Drs. A. V. Furano and C. M. Cashel [CH:LMG])

Histones

In collaboration with Dr. M. Klagsbrun, peptide maps were prepared from purified radioactive histones (F1, $F2a_1$, $F2a_2$, F2b, and F3) obtained from normal mouse cells (3T3) and their transformed counterparts (SV 3T3). The peptide maps of any one histone was the same regardless of source except for F3. In this case one peptide was missing from the map of 3T3 and replaced by a new one in SV 3T3, suggesting histone F3 might have been modified in the transformed cells. Work is underway to determine the nature of the modification.

(Drs. A. V. Furano and M. Klagsbrun)

Protein Methylation

In collaboration with Dr. M. Klagsbrun the nature of protein bound methylated amino acids in the various subcellular fractions (*e.g.*, membrane, "soluble" protein, and ribosomal protein) of *E. coli* and mammalian cells is being determined. These studies are preliminary to those in which we hope to study the relationship, if any, between protein methylation and various aspects of cellular regulation (*e.g.*, changes in growth rate after "step up" in bacteria, transformed versus normal cells, etc.)

(Drs. A. V. Furano and M. Klagsbrun)

3T3 and SV3T3 tRNA

The methylation of *Escherichia coli* B transfer RNA *in vitro* by HeLa mitochondrial and cytoplasmic extracts was examined. The following differences were found. (a) The cytoplasmic extract methylated *E. coli* B tRNA to a much greater extent than did the mitochondrial extract. (b) The two extracts methylated different sites on *E. coli* B tRNA, thereby generating different methylated oligonucleotide sequences. (c) The methylated base composition of *E. coli* B tRNA methylated *in vitro* indicated that 2-methyladenine tRNA methylase activity was uniquely associated with the mitochondria, while N^2-dimethylguanine tRNA methylase activity was uniquely associated with the cytoplasm. The presence of 2-methyladenine tRNA methylase activity in HeLa mitochondria and its absence in HeLa cytoplasm was taken as evidence for the existence of at least one mitochondrial-specific methylase. In addition, since 2-methyladenine is commonly found in prokaryote tRNA but not in eukaryote cytoplasmic tRNA, it was suggested that the presence of 2-methyladenine tRNA methylase activity in mitochondria was evidence that mitochondria might have evolved from prokaryote cells.

The methylated bases and nucleosides of transfer RNA and ribosomal RNA isolated from a number of eukaryote and prokaryote organisms were compared. Of the many different types of methylated bases and nucleosides found, only certain ones ever appeared in each type of methylated RNA; thus, the methylation of tRNA and both types of rRNA was a highly specific process. In addition, in all cells tested the pattern of methylation for tRNA and each rRNA differed markedly from each other.

Eukaryote tRNA, whether isolated from yeast, amoeba, chicken, mouse, monkey, or human cells, always exhibited a strikingly similar pattern of methylation. In addition, a second and third pattern of methylation were found to be common to the lower and higher molecular weight species of ribosomal RNA, respectively, in the eukaryote cells tested. Each of these three eukaryote RNA species contained at least one characteristic methylated base not found (or found only in trace amounts) in any of the others. These were N^2-dimethylguanine and 1-methylhypoxanthine in tRNA, N^2-dimethyladenine in 18 S RNA (16 S in yeast), and 3-methyluracil in 28 S RNA (25 S in yeast).

The patterns of methylation of prokaryote tRNA and rRNA were different from those of their eukaryote RNA counterparts. Two gram-negative bacteria, *E. coli* B and *Pseudomonas aeruginosa*, had identical methylation patterns for tRNA, 16 S RNA, and 23 S RNA, respectively. In addition, each of the ribosomal RNA species had a characteristic methylated base not found in the other. These were N^6-dimethyladenine in 16 S RNA, and N^6-methyladenine in 23 S RNA. The pattern of methylation of tRNA from gram-negative bacteria, gram-positive bacteria, and mycoplasma differed only slightly from each other.

It was concluded that great differences exist in the methylation patterns of the various RNA species in a given cell while great similarities exist in the methylation patterns of each RNA species throughout a wide range of eukaryote or prokaryote cells.

(Dr. M. Klagsbrun)

VI. TRAUMATIC SHOCK AND TRANSPLANTATION

Because of the action of phytohemagglutinin in cellular immunity, we are interested in obtaining a pure substance that is mitogenic for lymphocytes. Goldberg, Rosenau, and Burke reported that the mitogenic substance isolated from the red kidney bean, *Phaseolus vulgaris*, was not a glycoprotein, as generally believed, but a small molecular weight substance adsorbed to protein. In order to settle this controversy, we isolated a mitogenic substance by their procedure. This substance proved to be a glycoprotein, and not a small molecule. The glycoprotein is homogeneous, has a molecular weight of 121,000, and contains four subunits of equal size. The carbohydrate content of the molecule is 11.6%, of which 8.5% is mannose, 2% glucosamine, and 0.9% mannosamine.

In studies on the effect of trauma on skin allograft survival, a ring of Michele clips was used to fix the skin graft to its surrounding tissue. This is a commonly used technique. The usual procedure is to remove the clips seven days after grafting. We found that if the clips are left in place permanently, there is a striking prolongation of skin allograft survival time. The mechanism of this effect is not known, but one can speculate that this result is due to trauma with release of local and systemic immunosuppressive factors.

(Dr. K. Markley, Mrs. E. T. Smallman, and Mr. S. W. Thornton)

The influence of age and drugs was studied on local swelling of the mouse tail and ear produced by decreased local atmospheric pressure. Local swelling in the ear and tail decreased significantly during the time the animal was gaining weight most rapidly. Epinephrine was the only drug studied that decreased swelling in the mouse tail, but it had no effect on swelling of the ear.

(Dr. S. M. Rosenthal)

VII. MYCOBACTERIUM

Current studies on the growth of granulocytic colonies of mouse bone marrow require an agar medium and a colony-stimulating factor (CSF). Our new method omitted both the agar and CSF. However, the colonies were found to grow on top of a large cell which acted as if it were a "natural feeder cell." Cytochemical and cinemicrographic studies revealed that the feeder cell was probably the reticular cell of the bone marrow. Since the feeder cell was the only cell in culture which revealed strong alkaline phosphatase activity, it was postulated that this enzyme might be related to the colony-stimulating activity.

Among a total of 16 inbred strains of mice, the CBA/J mice showed the least animal-to-animal variations on the growth of intraperitoneally infected murine leprosy. By using the survival time of CBA/J mice as the criterion for drug evaluation, the significance of drug activity was able to be analyzed statistically. This appears to be the first model in mice in which a statistical analysis can be made satisfactorily.

(Dr. Y. T. Chang and Mrs. R. N. Andersen)

VIII. EVOLUTION

An experimental program to obtain evidence for our previously elaborated theory on the origin of the genetic code has been undertaken. A positive early result shows that the amino acids in the vertical uracil column of the codon table are arranged in order of decreasing hydrophobicity, Phe, Leu, Ile, Met, Val, as the theory assumes.

(Drs. S. Black and H. M. Chao)

Serial No. NIAMDD-LBP-1
1. Biochemical Pharmacology
2. Biochemistry of Amino Acids
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Biochemistry of Sulfur-Containing Compounds

Previous Serial Number: Same

Principal Investigator: Dr. Simon Black

Other Investigators: Dr. Helen M. Chao and Mrs. Blondel Hazel

Cooperating Units: None

Man Years:

Total:	4.1
Professional:	2.9
Others:	1.2

Project Description:

Objectives:

A long term objective is discovery of enzymatic and regulatory mechanisms involved in the synthesis of constituents of living tissue, particularly of proteins.

Methods Employed:

Chemical and enzymatic techniques involving the use of spectrophotometry and radioactive substances are evolved to meet the needs of special problems as they arise.

Major Findings:

I. Protein Synthesis. The glycyl tRNA synthetase from yeast, an essential enzyme in protein biosynthesis, catalyzes the following reaction:

$$\text{glycine} + \text{ATP} + \text{tRNA} \rightleftarrows \text{glycyl tRNA} + \text{AMP} + \text{PP}.$$

It has been found previously that when this enzyme is tested in the presence of inorganic sulfide, its activity becomes completely dependent upon two activator-like substances: glutathione and an alkali-denatured protein. It is our hypothesis that the denatured protein acts as a substitute for a specific activator present in the cell. During the past year evidence has been found that the sulfide-activated mechanism is of general importance, for it occurs in at least six other amino acyl tRNA synthetases from yeast and one from rat liver. The possibility that the mechanism is artifactual has been minimized by demonstrating it in fresh extracts made in several different ways. It has further been found that a sulfide-labile form of the enzyme can be stabilized by incubation in a crude cell-free extract, presumably by association with the activating factor discussed in previous years. In this light the previous tests were inadequate for excluding nonspecific effects.

II. Evolution. A theory was previously conceived for the origin of the genetic code. It has been extended to account for the origin of DNA and for the occurrence in DNA of thymine instead of uracil, which is found in RNA.

Experiments on the origin of the code have produced evidence that the hydrophobic amino acids of the vertical uracil column in the genetic codon table, phenylalanine, leucine, isoleucine, methionine, and valine, are arranged in descending order on the basis of their relative hydrophobicity.

Significance to Biomedical Research and the Program of the Institute:

I. Protein Synthesis. Elucidation of the nature of the enzyme's activation mechanism would open to similar study the regulation of many other of the more complex anabolic reactions of the cell, and reveal basic processes in the control of growth.

II. Evolution. It is hoped that a plausible theory for the origin of living organisms, supported by observation and experiment, will throw light on the nature of fundamental chemical interactions in present-day cells, and that this will be useful in enlarging our understanding and control of disease processes.

Proposed Course:

I. Protein Synthesis. Studies will continue on the activation mechanism of amino acyl tRNA synthetases.

II. Evolution. Theoretical and experimental work are being pursued with a view to determining how the first proteins and nucleic acids arose in nature, and how the genetic code originated.

Honors and Awards: None

Publications:

Black, S.: A theory on the origin of life. *Advances in Enzymology and Related Areas of Molecular Biology* 38: 193-234, 1973.

Serial No. NIAMDD-LBP-2
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Chemotherapy of Mouse Leprosy

Previous Serial Number: Same

Principal Investigator: Dr. Yao Teh Chang

Other Investigator: Mrs. Roxanna N. Andersen

Cooperating Unit: Leonard Wood Memorial (The American Leprosy Foundation)

Man Years:

Total:	2.3
Professional:	1.2
Other:	1.1

Project Description:

Objectives:

The evaluation of therapeutic effectiveness of drugs in mouse leprosy. The tissue cultures of intracellular parasites.

Methods Employed:

Chemotherapeutic studies were performed in mice infected intraperitoneally or into foot pads with *Mycobacterium lepraemurium*. Further attempts to cultivate *M. leprae* were made using cultures of human blood macrophages and a pure line of mouse bone marrow macrophages derived in our laboratory. Cytochemical and cinemicrographic studies of granulocytic colonies and macrophages of mouse bone marrow cells were performed in cultures in Leighton tubes and Sight chambers.

Major Findings:

I. Cultivation of Mycobacterium leprae in Macrophages. Human blood macrophages were able to maintain in good condition for about 2 months by an addition of dihydrocortisone in the culture medium. A pure cell line of macrophages was obtained by continued cultivation of mouse marrow cells for more than 3 years. These macrophages can be maintained in good condition at lower temperature, 30°, and also in a synthetic medium without serum or embryo extract. These factors are believed to be in favor for the growth of *M. leprae*. Trials on cultivation of *M. leprae* in both the human macrophages and the mouse macrophage line cells are in progress.

II. Improved Method for Chemotherapy of Murine Leprosy. Among a total of 16 inbred strains of mice, the CBA/J mice showed the least animal-to-animal variations on the growth of intraperitoneally infected murine leprosy. By using the survival time of animals as the criterion for drug evaluation, the significance of drug activity was able to be analyzed statistically. This appears to be the first model in mice in which statistical analysis can be applied satisfactorily. DDS showed a suppressive activity in a dose of 0.001% in the diet, which is 50 times lower than the effective dose observed with other screening methods.

III. Animal-to-Animal Variations on the Growth of M. lepraemurium and M. leprae. Contrary to the above findings, marked animal-to-animal variations of growth were observed when the organisms were injected into the foot pads. These variations were observed in a total of 16 inbred strains of mice infected with *M. lepraemurium*, and one strain (CBA/J) with *M. leprae*. DDS in a dose of 0.01% in the diet was not effective ($p > 0.10$) in both infections.

The importance of animal-to-animal variations has been ignored in the current *M. leprae* - foot pad studies. Only a small number of 4 foot pads were used for the tests and the results have not been analyzed statistically. DDS was claimed to be effective in a dose as small as 0.00001% in the diet which was calculated to be equivalent to 1 mg per patient. Clinical trials with this small dose of DDS has led to the development of DDS-resistant organisms in patients in many hospitals. This indicates that the screening method of the current *M. leprae* - foot pad technique is not accurate, and further studies, at least on the animal-to-animal variations of growth, are needed.

IV. Cytochemical Studies in the "Natural Feeder Cell" of Granulocytic Colonies. The feeder cell revealed strong alkaline phosphatase activity, suggesting that the feeder cell was probably related to the reticular cell of bone marrow. Since the feeder cell was the only cell in the culture showing alkaline phosphatase activity, it was postulated that this enzyme might be related to the growth of the colonies.

V. Cinemicrographic Studies of the Growth of Granulocytic Colonies. Current studies on the growth of granulocytic colonies limited to a period of only 6-7 days, after which the colonies were replaced by tremendous growth of mononuclear cells. Our studies revealed a total of 3 stages of colony growth. Many colonies were observed in the first 6-7 days. Then, new colonies appeared which last for 5-6 days. A few colonies were again observed at the 12th day and lasted for another 5-6 days. Death of colony cells was observed around the 12th and 17th days. This may be the main cause for the termination of growth of granulocytic cells in the cultures.

VI. A Device Was Developed for Eliminating Oil Condensation in the Air Flowmeter of CO_2 Incubator. Oil condensation in the air flowmeter has been a problem in many laboratories. This problem was solved by installing a metal needle-valve in the air line, after which oil vapor will be condensed on the metal valve and the metal connections. The condensed oil can be drained through another valve into a test tube. This system has been used in this laboratory for more than 10 years without oil accumulation in the air flowmeter.

Significance to Biomedical Research and the Program of the Institute:

The human macrophages and the macrophage line of mouse marrow cells may be considered as hopeful candidates as the host cells for cultivation of *M. leprae*. The improved method for quantitative evaluation of drug activity in murine leprosy will be a useful tool for future chemotherapeutic studies. The identification of the "natural feeder cell" of granulocytic colonies as a reticular cell offers a fundamental understanding of granulopoiesis. Pathologists have been trying to establish the relationship between reticular cell and hemopoiesis. The present finding furnishes an experimental evidence to support such an hypothesis. The device for eliminating oil condensation in the air flowmeter in a CO_2 incubator is of practical value. This has been a problem in many laboratories, including the NIH Clinical Center, where the air lines of several floors were contaminated by oil.

Proposed Course:

Studies will be continued toward the development of techniques for cultivation of human macrophages, on the growth of *M. leprae* therein, on cytochemical and cinemicrographic studies of host-parasite relationship, in *in vitro* granulocytopoiesis, and on development of a more accurate model for drug screening in murine leprosy.

Honors and Awards:

My term of service of 5 years as a member of the Expert Advisory Panel on Leprosy of the World Health Organization will expire in July 1973. An invitation has been received to accept an extension of membership for a further period of 5 years.

Publications:

Chang, Y. T.: Device for eliminating oil condensation in the air flowmeter in CO_2 incubator. *Applied Microbiology* 24: 840, 1972.

Serial No. NIAMDD-LBP-3
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies in Traumatic Shock and Cellular Immunity

Previous Serial Number: Same

Principal Investigator: Dr. Kehl Markley

Other Investigators: Dr. Sanford M. Rosenthal and Mrs. Elizabeth T. Smallman

Cooperating Units: None

Man Years

Total: 3.1
Professional: 2.3
Others: 0.8

Project Description:

Objectives:

I. To obtain and characterize a purified mitogen from the red kidney bean, *Phaseolus vulgaris*.

II. To study new methods to prolong skin allograft survival time.

III. To study the influence of age and drugs on local swelling of the mouse tail and ear produced by decreased local atmospheric pressure.

Methods Employed:

I. The mitogen was isolated from *Phaseolus vulgaris* by the method of Goldberg, Rosenau, and Burke. To purify further, the final product was concentrated and placed on a Sephadex G-200 column. Mitogenic activity was assayed by the incorporation of [^{3}H]thymidine into the DNA of peripheral human lymphocytes. The mitogen was characterized for purity by disc gel electrophoresis and sedimentation velocity and equilibrium experiments. The molecular weight was determined by sedimentation equilibrium, and subunit size by disc gel electrophoresis in the presence of sodium dodecylsulfate. The amino acid content was calculated from data obtained on the Beckman amino acid analyzer. Cysteic acid was measured after performic acid oxidation and S-carboxymethylcysteine after alkylation.

The mitogen was digested by pronase and trypsin after mild heating. The carbohydrate content was studied by standard procedures for hexose and hexosamine.

II. The basic grafting technique used was described by Hauschka, Grinnell, Meagher, and Amos. This method consists of applying a ring of Michele clips to fix the skin graft to its surrounding tissue. Seven days after grafting, the clips are usually removed. Variations on this technique include: (1) placing no clips but using sutures instead; (2) using only 6 clips to fix the graft; (3) allowing the clips to remain in place permanently; (4) placing the clips 0.5-1 cm away from the edge of the graft; and (5) placing the clips 1-3 cm distant from the graft. The effect of these variations was studied on skin graft survival time.

III. Decreased local atmospheric pressure was applied to the mouse tail as described previously to produce local edema. A similar method was developed to study swelling of the mouse ear. NIH female mice of various age groups were utilized, and drugs were injected intraperitoneally prior to subjecting the mice to the reduced pressure.

Major Findings:

I. Goldberg, Rosenau, and Burke reported that the mitogenic substance isolated from the red kidney bean, *Phaseolus vulgaris*, was not a glycoprotein, as generally believed, but a small molecular weight substance adsorbed to protein. In order to settle this controversy, we isolated a mitogenic substance by their procedure. This substance proved to be a glycoprotein, and not a small molecule. The glycoprotein is homogeneous, has a molecular weight of 121,000, and contains four subunits of equal size. The carbohydrate content of the molecule is 11.6%, of which 8.5% is mannose, 2% glucosamine, and 0.9% mannosamine.

II. Skin graft survival time was markedly prolonged by keeping the Michele clips in place permanently. Studies on variations of the grafting technique did not elucidate the mechanism of this effect, but they did suggest that the result is due to trauma with release of local and systemic immunosuppressive factors.

III. Local swelling in the ear and tail decreased significantly during the time the animal was gaining weight most rapidly. Epinephrine was the only drug studied that decreased swelling in the mouse tail, but it had no effect on swelling of the ear.

Significance to Biomedical Research and the Program of the Institute:

I. Phytohemagglutinin obtained from the red kidney bean is an important agent in the study of the biochemical events associated with cellular division. Many of the biologic effects ascribed to phytohemagglutinin from *Phaseolus vulgaris* have been obtained with impure preparations of phytohemagglutinin. There is a great need for purified preparations to test which effect is produced by which component of the preparation. We have purified a mitogenic glycoprotein and described its physical and chemical properties.

II. The finding that the survival time of skin allografts can be markedly prolonged by variations in the grafting technique is important when evaluating immunosuppressive agents. These results are also interesting from their theoretical implications on cellular immunity.

III. The studies on the effect of age and drugs on swelling produced by reduced atmospheric pressures has significance on the understanding of the physiologic mechanism of edema formation.

Proposed Course:

I. To alter the chemical structure of the mitogen of phytohemagglutinin and to study the changes in its biological effects.

II. To search for chemical mediators produced by trauma which alter the cellular immune response.

III. To continue the study of the mechanism of edema formation.

Honors and Awards: None

Publications:

Markley, K., Smallman, E., Thornton, S. W., and Evans, G.: The effect of environmental temperature and fluid therapy on mortality and metabolism of mice after burn and tourniquet trauma. *J. Trauma* 13: 145-160, 1973.

Shapiro, H. M., Markley, K., and Smallman, E.: Modification of burn shock mortality in mice by an oxidizing and a reducing agent. *Proc. Soc. Exp. Biol. Med.* 142: 968-972, 1973.

Serial No. NIAMDD-LBP-4
1. Biochemical Pharmacology
2. Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Biology of Complex Carbohydrates

Previous Serial Number: Same

Principal Investigator: Dr. Victor Ginsburg

Other Investigators: Drs. David A. Zopf, Carol A. Mapes, Alan M. Jeffrey, and Chaviva Isersky-Carter

Cooperating Units: None

Man Years:

Total:	5.0
Professional:	4.3
Others:	0.7

Project Description:

Objectives:

To investigate the role of complex carbohydrates in cell biology.

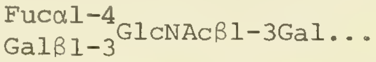
Methods Employed:

Usual biochemical techniques.

Major Findings:

Specific sugar sequences found in the free oligosaccharides of human milk also occur in glycoproteins and glycolipids of cell surfaces and are important determinants of immunologic specificity. Some sequences predominate in certain human tumors as contrasted to corresponding normal tissue where they are either absent or present at low levels. Antisera directed against these sequences should be useful diagnostically as evidenced by the importance of the carbohydrate "carcino-embryonic" antigen described by Gold and Freedman for the detection of human colonic carcinomas. We are preparing antibodies against pure milk oligosaccharides for possible use in the detection of "tumor antigens" and for studies on changes in cell surface sugars during malignant transformation.

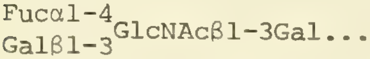
Free oligosaccharides are not antigenic themselves and first have to be coupled to carriers. Published procedures for effecting this coupling are either too harsh for milk oligosaccharides with labile linkages or have a yield of product too low for use with the rarer sugars. For this reason we have investigated several methods for coupling the pentasaccharide, lacto-*N*-fucopentaose II (LNF-II) and have tested the coupled products for antigenicity in rabbits. LNF-II contains the nonreducing terminus:



$$\begin{matrix}\text{Fuc}\alpha 1\text{-}4\\ \text{Gal}\beta 1\text{-}3\end{matrix}\text{GlcNAc}\beta 1\text{-}3\text{Gal}\ldots$$

Two of the methods tested were quite successful: LNF-II was oxidized with I_2 in 95% yield to a gluconic acid derivative. The oxidized LNF-II was then coupled with carbodiimide in 80% yield to Cellex PAB. The oxidized LNF-II was also coupled in high yield to bovine serum albumin using the mixed anhydride technique of Ashwell. Although in the latter technique only 20% of the product is soluble while the remainder precipitates, both the soluble and insoluble material proved to be effective antigens.

Rabbits immunized by standard methods with the Cellex PAB and bovine serum albumin derivatives of LNF-II all developed antibodies against the structure



$$\begin{matrix}\text{Fuc}\alpha 1\text{-}4\\ \text{Gal}\beta 1\text{-}3\end{matrix}\text{GlcNAc}\beta 1\text{-}3\text{Gal}\ldots$$

From 0.1 to 0.4 mg of precipitable antibody protein per ml of serum was produced in 9 rabbits as measured by quantitative precipitin assays using as antigen either an LNF-II derivative or human Le^a blood group substance which contains $\begin{matrix}\text{Fuc}\alpha 1\text{-}4\\ \text{Gal}\beta 1\text{-}3\end{matrix}\text{GlcNAc}\beta 1\text{-}3\text{Gal}\ldots$ groupings. Antibody specificity was determined by hapten inhibition of precipitation using LNF-II and related oligosaccharides. For example, LNF-II inhibited 50% at 0.7 μmole/ml and 95% at 5 μmoles/ml. Its closely related isomer LNF-I which has the structure Fucα1-2GlcNAcβ1-3Gal..., had no effect at 12 μmoles/ml. LNT, which has the the structure Galβ1-3GlcNAcβ1-3Gal..., inhibited 50% at 7 μmoles/ml while free fucose inhibited 50% at 30 μmoles/ml.

The rabbit antibody against LNF-II was purified by affinity chromatography using LNF-II coupled to "affinose" as absorbant. Antibody was eluted by fucose from the column in high yield and purity.

Significance to Biomedical Research and the Program of the Institute:

These studies may contribute to an understanding of cell-cell interactions which are important to biological phenomena such as graft rejection, autoimmune diseases, and carcinogenesis.

Proposed Course:

We will continue to try to elucidate the carbohydrates on cell surfaces that are responsible for their specificity.

Honors and Awards: None

Publications:

Ginsburg, V.: Biochemical basis for blood groups in man. In Pontis, H. G., and Piras, R. (Eds.): *Biochemistry of the Glycosidic Linkage, PAABS Symposium*, Vol. 2. New York, New York, Academic Press, 1972, pp. 387-393.

Ginsburg, V.: Enzymatic basis for blood types in man. In Meister, A. (Ed.): *Advances in Enzymology and Related Areas of Molecular Biology*, Vol. 36. New York, New York, John Wiley & Sons, 1972, pp. 131-149.

Ginsburg, V. (Ed.): *Methods in Enzymology - Complex Carbohydrates*. New York, New York, Academic Press, 1972, Vol. 28, 1,057 pp.

Kobata, A.: An *N*-acetylgalactosaminyltransferase from human milk: a product of the gene that determines blood type A. In Ginsburg, V. (Ed.): *Methods in Enzymology - Complex Carbohydrates*, Vol. 28. New York, New York, Academic Press, 1972, pp. 511-514.

Kobata, A.: Isolation of oligosaccharides from human milk. In Ginsburg, V. (Ed.): *Methods in Enzymology - Complex Carbohydrates*, Vol. 28. New York, New York, Academic Press, 1972, pp. 262-271.

Kobata, A., and Ginsburg, V.: Oligosaccharides of human milk. IV. Isolation and characterization of a new hexasaccharide, lacto-*N*-*neo*hexaose. *Arch. Biochem. Biophys.* 150: 273-281, 1972.

Shen, L. C., and Ginsburg, V.: Sugar analysis of cells in culture by isotope dilution. In Ginsburg, V. (Ed.): *Methods in Enzymology - Complex Carbohydrates* Vol. 28. New York, New York, Academic Press, 1972, pp. 195-201.

Serial No. NIAMDD-LBP-5
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Estimation, Metabolism, and Function of Amines

Previous Serial Number: NIAMDD-LBP-5A

Principal Investigators: Drs. Herbert Tabor and Celia White Tabor

Other Investigators: Dr. Hitoshi Fujisawa and Mrs. Patricia Deal Kellogg

Cooperating Units: Dr. Filadelfo Irreverre, Laboratory of Biophysical Chemistry, NIAMDD

Man Years:

Total:	4.7
Professional:	3.2
Other:	1.5

Project Description:

Objectives:

The importance of the various polyamines is shown by their wide distribution in viruses, bacteria, plant cells, and animal cells. Studies are being conducted to elucidate their metabolism and function.

Methods Employed:

The amines and their derivatives are studied by isotopic labeling and special chromatographic techniques. Standard enzymological techniques have been used to purify further the enzymes that synthesize and degrade spermine, spermidine, and their derivatives. Chemical synthetic procedures have been utilized to prepare derivatives for identification of enzymatic products. Uptake of amines was studied by counting the residual radioactivity in washed pellets on Millipore filters. Gel electrophoresis techniques have been utilized to study the ribosome pattern of cells. Amino acid analyses were carried out in an automatic amino acid analyzer. Fluorometric analyses have been utilized.

An amino acid analyzer has been utilized for the development of a new, automated, sensitive, and quantitative method for the analysis of amines and amine derivatives.

The metabolism of glutathione in *Escherichia coli* is being studied both with isotopic techniques and by the development of an automated system of analysis, using an amino acid analyzer.

Major Findings:

A sensitive and specific method for the automated analysis of polyamines has been further developed and improved in collaboration with Dr. F. Irreverre [A:LBC]. This method is being used to study the amine patterns in bacteria and mammalian cells. We are continuing our studies of enzymes involved in amine biosynthesis and degradation, with emphasis on the propylamine transferase that carries out the synthesis of spermidine in *Escherichia coli*, a new peptidase in *E. coli* that splits glutathionylspermidine to free spermidine, and the spermidine dehydrogenase of *Serratia marcescens* which acts on glutathionylspermidine as well as on free spermidine.

The metabolism of glutathione and other sulfhydryl compounds found in the trichloroacetic acid extracts of *E. coli* is of particular interest because of the conversion of all of the bacterial spermidine and half of the glutathione to a covalent derivative when the bacteria culture approaches the stationary phase. Substantial amounts of this material have been isolated. The structure has been shown to be glutathionylspermidine, in which the glycine carboxyl is linked to spermidine.

A derivative of putrescine, hydroxyputrescine, has been found by several laboratories in a mutant strain of *Pseudomonas*. Large amounts are also found in a wild type *Pseudomonas* (approximately 1 μmole/gram wet weight, an amount about equal to the putrescine content of these cells). However, many other pseudomonads have only 10-20% as much hydroxyputrescine. We have thus far not found it in *Escherichia coli*, *Serratia marcescens*, or *Bacillus subtilis*. The hydroxyputrescine is derived from putrescine *in vivo*, when cells are labeled with [^{14}C]putrescine, and preliminary studies indicate that there is no degradation of hydroxyputrescine by *Pseudomonas*.

A new, unstable RNA fraction has been observed in extracts of *E. coli*. We are studying the nature of this material, using acrylamide gel electrophoresis, density gradient centrifugation, and isotopic methods. The preliminary evidence indicates that this material is smaller than 4 S RNA and can be made to accumulate under special conditions of lysis.

Significance to Biomedical Research and the Program of the Institute:

Recent work has indicated that the polyamines may play an important role in permeability of organisms and in the stability of nucleic acids inside cells. Studies by Pardee and Maas suggest that the amines may also play an important role in the division of *E. coli*.

Some of the polyamines have also been found to act as growth factors for bacteria, plants, and certain mammalian cell lines in culture. Our studies of the amine content and metabolism in bacteria and mammalian cells under various growth conditions will contribute to our understanding of the functions of these ubiquitous compounds.

Our studies on the propylamine transferase enzyme may lead to elucidation of the mechanism of the formation of the C-N bond in bacteria and the role of the high-energy sulfonium ion in this reaction.

Proposed Course:

Further studies on the metabolism and function of the biologically important polyamines. We hope to learn the normal physiological function of these amines in the cell and to explain such pathological effects as the production of renal damage by spermine.

Honors and Awards: None

Publications:

Bowman, W. H., Tabor, C. W., and Tabor, H.: Spermidine biosynthesis: purification and properties of propylamine transferase from *Escherichia coli*. *J. Biol. Chem.* 248: 2480-2486, 1973.

Tabor, H., and Tabor, C. W.: Biosynthesis and metabolism of 1,4-diaminobutane, spermidine, spermine, and related amines. In Meister, A. (Ed.): *Advances in Enzymology and Related Areas of Molecular Biology*, Vol. 36. New York, New York, John Wiley & Sons, 1972, pp. 203-268.

Tabor, H., Tabor, C. W., and Irreverre, F.: Quantitative determination of aliphatic diamines and polyamines by an automated liquid chromatography procedure. *Anal. Biochem.*, 1973, in press.

Serial No. NIAMDD-LBP-6
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Enzymatic Studies of Nucleic Acid Metabolism

Previous Serial Number: Same

Principal Investigator: Dr. Nancy G. Nossal

Other Investigator: Dr. Frances D. Gillin

Cooperating Units: None

Man Years:

Total:	2.2
Professional:	1.7
Other:	0.5

Project Description:

Objectives:

To determine the characteristics and functions of enzymes which are involved in the synthesis, modification, and degradation of DNA.

Methods Employed:

Standard biochemical techniques were employed for the preparation of polynucleotide substrates and enzymes. Extensive use has been made of amber mutants of T4 bacteriophage which are unable to make specific early enzymes in nonpermissive hosts. Procedures have been developed for the large scale preparation of these amber mutants and for the production of kilogram quantities of infected bacterial cells.

Major Findings:

I. DNA Synthesis by the T4 Polymerase in the Presence of the Gene 32 DNA Unwinding Protein. The DNA polymerase induced by T4 bacteriophage is one of several proteins which are absolutely required for the replication of the phage DNA *in vivo*. This polymerase has both polymerase and 3' to 5' exonuclease activities in a single polypeptide of MW 110,000. Genetic and biochemical studies with mutants in the structural gene for this enzyme suggest that this polymerase is involved in the replication of the linear duplex DNA of the virus. *In vitro*, however, the enzyme is unable to copy double stranded DNA, and its copying of single stranded DNA is inefficient compared with its rate *in vivo*, in part because its exonuclease activity carries out extensive hydrolysis of newly incorporated residues. Thus it appears likely that the addition of other phage induced proteins will be necessary in order to reconstruct a system capable of copying native duplex DNA *in vitro*.

As a first step toward this goal, we have purified the gene 32 protein to a point where it is both homogeneous and free of contaminating nucleases. This protein has been shown by Alberts and his collaborators to be required in stoichiometric amounts for T4 DNA synthesis *in vivo* and to partially unwind DNA and stimulate the rate of DNA synthesis using single stranded templates with the T4 polymerase *in vitro*. We have used T7 DNA as a model for the copying of linear duplexes, since it is small enough to be prepared in a homogeneous form. We have previously shown that the T4 polymerase by itself cannot catalyze the stable incorporation of triphosphates into product with this template, although there is extensive incorporation and subsequent hydrolysis of residues at the ends of the duplex chains. In contrast, if the 32 protein is present, the T4 polymerase can use the native duplex as a template, making a quantity of product approaching that of the template. Preliminary studies suggest that the product is not covalently attached to the template, since if BrdUTP is used as a density label, most of the product bands in alkaline CsCl at the same position as "heavy" single strands of DNA, while most of the template bands with "light" single strands. Some product and template do band together at a position expected for strands containing light and heavy residues. In neutral CsCl the template and product band together at the hybrid density. The size distribution of the template and product appear to be similar since they sediment together in both neutral and alkaline sucrose gradients. Under the same conditions where stable copying of native T7 DNA is achieved, the turnover of residues at the chain ends is stimulated less than two-fold over that occurring in the absence of the 32 protein.

We have used the synthetic polymer poly d(AT) as a model for the copying of single stranded DNA, since the intrastrand binding of this polymer is efficiently unwound by the gene 32 protein at low temperatures. We find that the T4 polymerase catalyzes the several-fold net synthesis of this polymer in the presence of the gene 32 protein. Synthesis continues until the available deoxynucleoside triphosphates have been depleted. In the absence of the 32 protein, the rate of synthesis of poly d(AT) is much slower and extent of product formed does not exceed a single copy.

(Dr. N. G. Nossal)

II. Control of Mutation Rates by the T4 DNA Polymerase. The T4 DNA polymerase offers a good system in which to study factors controlling the fidelity of DNA replication. Some mutations in the structural gene for the DNA polymerase (gene 43) which are temperature sensitive for DNA replication and phage production, at permissive temperatures show either an increased or decreased rate of mutation of markers in other genes. We have purified the DNA polymerase induced by the phage mutant, *ts* L88, which has a greatly increased rate of mutation compared to the wild type *in vivo*, and have compared the frequency of *in vitro* copying errors of the mutant and wild type enzymes.

The *ts* L88 mutant enzyme uses the wrong nucleotide (either dGTP or dCTP) at a higher frequency than the wild type enzyme when poly (dA) · poly (dT) is used as the template primer. With both the mutant and the wild type enzymes, the noncomplementary residues are subsequently completely removed by the exonuclease activities associated with the enzymes. Thus the mutator enzyme appears to differ from the wild type in the frequency with which its polymerase incorporates a noncomplementary base, rather than in the efficiency of its exonuclease in removing a misincorporated residue. It seems likely that *in vivo* the ratio of polymerase to nuclease activity is higher than that which we observe *in vitro*, so that some of the misincorporated residues would escape the editing of the nuclease. This suggestion is supported by the fact that polymerase activity is stimulated by the gene 32 protein to a far greater extent than is the hydrolysis of newly incorporated residues. The temperature sensitive phenotype of the *ts* L88 enzyme also appears to arise from a change in polymerase rather than nuclease activity. Thus the specific activity and thermolability of nuclease are similar for both enzymes, but the mutant specific activity for polymerase is lower and its thermoability much greater than that of the wild type enzyme.

We have also purified the DNA polymerase from *ts* CB120, a T4 mutant in gene 43 which *in vivo* has a very low frequency of mutation. This mutant enzyme has about one-sixth the specific activity for polymerase as the wild type enzyme, while the specific activities for nuclease of the two enzymes are equal. Neither activity is more heat-labile in the mutant than in the wild type enzyme. A larger fraction of the newly incorporated residues are hydrolyzed by the *ts* CB120 enzyme than by the wild type, even in the presence of the 32 protein. We are currently trying to determine whether this increased nuclease activity is responsible for the low mutation rate, or whether there is also a decrease in the incorporation of noncomplementary nucleotides by this enzyme.

(Drs. M. S. Hershfield and F. D. Gillin)

III. Cyclic AMP Levels during Sporulation of Bacillus subtilis. Sporulation of *B. subtilis*, chosen as a relatively simple example of cell differentiation, does not appear to be affected by cyclic AMP. We found that the low level of cyclic AMP present in *B. subtilis* (approximately 1 x 10^{-7} M) does not change during the process of sporulation, and that exogeneous cyclic AMP, dibutyryl cyclic AMP, or cyclic GMP do not influence the time course of sporulation.

(Dr. F. D. Gillin)

Significance to Biomedical Research and the Program of the Institute:

Knowledge of enzymes controlling DNA synthesis and degradation is important to an understanding of the control of DNA replication and transcription in both normal and viral infected cells.

Proposed Course:

The copying of native DNA catalyzed by the T4 DNA polymerase in the presence of the gene 32 unwinding protein will be further characterized to determine whether product chains are initiated *de novo* or are primed by DNA or RNA oligonucleotides. We will try to purify the products of other phage genes known to be required for DNA replication to test their effect on DNA synthesis *in vitro*. We will continue our study of the control of mutation rates by the T4 DNA polymerase.

Honors and Awards:

Dr. Nancy G. Nossal elected to the American Society of Biological Chemists in 1972.

Publications:

Hershfield, M. S.: On the role of deoxyribonucleic acid polymerase in determining mutation rates. Characterization of the defect in the T4 deoxyribonucleic acid polymerase caused by the *ts* L88 mutation. *J. Biol. Chem.* 248: 1417-1423, 1973.

Hershfield, M. S., and Nossal, N. G.: Hydrolysis of template and newly synthesized deoxyribonucleic acid by the 3' to 5' exonuclease activity of the T4 deoxyribonucleic acid polymerase. *J. Biol. Chem.* 247: 3393-3404, 1972.

Hershfield, M. S., and Nossal, N. G.: *In vitro* characterization of a mutator T4 DNA polymerase. *Genetics*, in press, 1973.

Nossal, N. G., and Hershfield, M. S.: Exonuclease activity of wild type and mutant T4 DNA polymerases. Hydrolysis during DNA synthesis *in vitro*. In Wells, R. D., and Inman, R. B. (Eds.): *DNA Synthesis in Vitro*. Baltimore, Maryland, University Park Press, 1973, in press.

Serial No. NIAMDD-LBP-7
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Protein Synthesis in *Escherichia coli*

Principal Investigator: Dr. Anthony V. Furano

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.1
Professional:	1.0
Others:	0.1

Project Description:

Objectives:

To study the mechanism and control of protein synthesis *in vivo* and *in vitro*. This laboratory is also involved in studying the processes and effects of protein modification (*e.g.*, methylation) in collaboration with Dr. Michael Klagsbrun [A:LBP].

Methods Employed:

In vivo. We have been studying a mutant of *E. coli* which cannot synthesize protein at 42°.

In vitro. We are studying the soluble enzymes that catalyze in some way polypeptide chain elongation.

Protein Modification (in collaboration with Dr. M. Klagsbrun). We are identifying the types of methylated amino acids present in the various subcellular fractions of *E. coli* and mammalian cells. We are also comparing the histones obtained from normal mouse fibroblasts grown in culture and from their transformed counterparts.

Major Findings:

Evidence obtained earlier in this laboratory has suggested that peptide chain elongation factors may be subject to proteolysis during purification.

Accordingly, a method of purification of these factors has been devised incorporating the use of the proteolytic inhibitor, phenylmethylsulfonyl chloride, and avoiding any steps which concentrate the proteins while they are still relatively impure. Using this technique, three polypeptides involved in peptide chain elongation have been isolated from cell-free extracts of *E. coli* these proteins have molecular weights of 47,500, 35,000, and about 83,000, referred to in this summary as A, B, and C, respectively. In terms of some chromatographic and electrophoretic properties, A, B, and C are analogous to "Tu", "Ts", and "G", respectively. However, A and B have molecular weights that are significantly greater than those reported for Tu (40,000) and Ts (30,000). In rapidly growing bacteria, A, B, and C make up a significant percentage (5-10%) of the nonparticulate protein, and A (47,500) is by far in the largest amount. The bulk of A exists free in the cytoplasm but some is bound noncovalently in a very stable complex to B. There is no evidence that B exists free in the cytoplasm. A, the A-B complex, and C (83,000) have been purified to homogeneity. Either A or A-B is required, along with C, for polypeptide synthesis *in vitro* with highly purified ribosomes; little or no polypeptide synthesis takes place in the absence of C or with C alone. These results suggest that the function provided by B (35,000) is not absolutely required under the conditions of the assay *in vitro*. Experiments are currently underway in this laboratory as well as in collaboration with Dr. C. M. Cashel [CH:LMG] to analyze the requirement and effect of GTP, the novel G tetra and pentaphosphate derivatives of GTP and other phosphorylated compounds on the reactions carried out by the elongation factors.

Experiments are also underway to examine whether there are differences between the A that is complexed with B and the one that is not, by comparing peptide maps prepared from purified A, the A-B complex, and C. The latter comparison might prove to be especially interesting because of the many similar chromatographic and electrophoretic properties between the A-B complex and C.

In collaboration with Dr. M. Klagsbrun, peptide maps were prepared from purified radioactive histones (Fl, F2a$_1$, F2a$_2$, F2b, and F3) obtained from normal mouse cells (3T3) and their transformed counterparts (SV 3T3). The peptide maps of any one histone was the same regardless of source except for F3. In this case one peptide was missing from the map of 3T3 and replaced by a new one in SV 3T3, suggesting histone F3 might have been modified in the transformed cells. Work is underway to determine the nature of the modification.

In collaboration with Dr. M. Klagsbrun, the nature of protein bound methylated amino acids in the various subcellular fractions (*e.g.*, membrane, "soluble" protein, and ribosomal protein) of *E. coli* and mammalian cells is being determined. These studies are preliminary to those in which we hope to study the relationship, if any, between protein methylation and various aspects of cellular regulation (*e.g.*, changes in growth rate after "step up" in bacteria, transformed versus normal cells, etc.)

Significance to Biomedical Research and the Program of the Institute:

Knowledge on the mechanism of protein synthesis and the processes and effects of protein modification (*e.g.*, methylation) is essential to understanding cellular metabolism and growth.

Proposed Course:

This work is currently in progress and will be continued and extended.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LBP-8
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1972

Project Title: The Methylation of RNA and Protein in Prokaryote and Eukaryote Organisms

Previous Serial Number: Same

Principal Investigator: Dr. Michael Klagsbrun

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.2
Professional:	1.0
Others:	0.2

Project Description:

Objectives:

To determine what biological role is served by the methylation of RNA and protein in animal and bacterial cells. The nature of this biological role is at present unclear.

Methods Employed:

Standard tissue culture and biochemical techniques such as column and thin layer chromatography, sucrose gradients, gel electrophoresis, and autoradiography.

Major Findings:

The methylation of *Escherichia coli* B transfer RNA *in vitro* by HeLa mitochondrial and cytoplasmic extracts was examined. The following differences were found. (a) The cytoplasmic extract methylated *E. coli* B tRNA to a much greater extent than did the mitochondrial extract. (b) The two extracts methylated different sites on *E. coli* B tRNA, thereby generating different methylated oligonucleotide sequences. (c) The methylated base composition of *E. coli* B tRNA methylated *in vitro* indicated that 2-methyladenine tRNA methylase activity was uniquely associated with the mitochondria, while N^2-dimethylguanine tRNA methylase activity was uniquely associated with the cytoplasm. The presence of 2-methyladenine tRNA methylase activity in HeLa mitochondria and its absence in HeLa cytoplasm was taken as evidence for the existence of at least one mitochondrial-specific methylase. In addition, since 2-methyladenine is commonly found in prokaryote tRNA but not in eukaryote cytoplasmic tRNA, it was suggested that the presence of 2-methyladenine tRNA methylase activity in mitochondria was evidence that mitochondria might have evolved from prokaryote cells.

The methylated bases and nucleosides of transfer RNA and ribosomal RNA isolated from a number of eukaryote and prokaryote organisms were compared. Of the many different types of methylated bases and nucleosides found, only certain ones ever appeared in each type of methylated RNA; thus, the methylation of tRNA and both types of rRNA was a highly specific process. In addition, in all cells tested the pattern of methylation for tRNA and each rRNA differed markedly from each other.

Eukaryote tRNA, whether isolated from yeast, amoeba, chicken, mouse, monkey, or human cells, always exhibited a strikingly similar pattern of methylation. In addition, a second and third pattern of methylation were found to be common to the lower and higher molecular weight species of ribosomal RNA, respectively, in the eukaryote cells tested. Each of these three eukaryote RNA species contained at least one characteristic methylated base not found (or found only in trace amounts) in any of the others. These were N^2-dimethylguanine and 1-methylhypoxanthine in tRNA, N^2-dimethyladenine in 18 S RNA (16 S in yeast), and 3-methyluracil in 28 S RNA (25 S in yeast).

The patterns of methylation of prokaryote tRNA and rRNA were different from those of their eukaryote RNA counterparts. Two gram-negative bacteria, *E. coli* B and *Pseudomonas aeruginosa*, had identical methylation patterns for tRNA, 16 S RNA, and 23 S RNA, respectively. In addition, each of the ribosomal RNA species had a characteristic methylated base not found in the other. These were N^6-dimethyladenine in 16 S RNA, and N^6-methyladenine in 23 S RNA. The pattern of methylation of tRNA from gram-negative bacteria, gram-positive bacteria, and mycoplasma differed only slightly from each other.

It was concluded that great differences exist in the methylation patterns of the various RNA species in a given cell while great similarities exist in the methylation patterns of each RNA species throughout a wide range of eukaryote or prokaryote cells.

Significance to Biomedical Research and the Program of the Institute:

The methylation of RNA, DNA, and protein results in the chemical modification of these macromolecules. These modifications may be of importance in regulating the biological functions of the macromolecules in a way which is yet not understood. It has been proposed, for example, that excess methylation of transfer RNA in tumor cells leads to aberrant protein synthesis in these cells. Therefore, a clearer understanding of the methylation process would be useful.

Proposed Course:

The methylation of various proteins in *E. coli* and in normal and SV40 transformed mouse cells will be explored in detail. Particular attention will be focused on the methylation of histones which are known to be involved in the regulation of DNA synthesis.

Honors and Awards:

Election to the American Society of Biological Chemists in 1973.

Publications:

Klagsbrun, M.: The contrast between the methylation of transfer ribonucleic acid *in vivo* and *in vitro* by normal and SV40 transformed 3T3 cells. *J. Biol. Chem.* 247: 7443-7451, 1972.

Klagsbrun, M.: An evolutionary study of the methylation of transfer and ribosomal ribonucleic acid in prokaryote and eukaryote organisms. *J. Biol. Chem.* 248: 2612-2620, 1973.

Klagsbrun, M.: Differences in the methylation of transfer ribonucleic acid *in vitro* by the mitochondrial and cytoplasmic transfer ribonucleic acid methylases of HeLa cells. *J. Biol. Chem.* 248: 2606-2611, 1973.

Klagsbrun, M., and Farrell, P. M.: The methylation of ribonucleic acid in fibroblasts of normal volunteers and patients with cystic fibrosis. In Mangos, J. A., and Talamo, R. C. (Eds.): *Fundamental Problems of Cystic Fibrosis and Related Diseases*. Miami, Florida, Symposia Specialists, 1973, *in press*.

Serial No. NIAMDD-LBP-9
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the Chemical and Physiological Properties of the Surface of *Escherichia coli*

Previous Serial Number: Same

Principal Investigator: Dr. Loretta Leive

Other Investigators: Drs. Charles F. Kulpa, Jr., Akira Kimura, and Pinakilal Bhattacharyya

Cooperating Units: None

Man Years:

Total:	3.3
Professional:	3.0
Other:	0.3

Project Description:

Objectives:

To study the chemical and physiological properties of the cell surface of *E. coli*.

Methods Employed:

Usual biochemical methods, including isolation and purification of cell surface components by extraction, ultracentrifugation, chromatography, and selective enzymatic degradation, determination of the size and shape of these fractions by biophysical measurements of density, viscosity, and ultracentrifugal properties, identification and quantitation of sugars by colorimetric and enzymatic procedures, and use of labeled compounds to specifically label cell fractions *in vivo*.

Major Findings:

Structure and Assembly of Escherichia coli Membranes. The surface of *E. coli* consists of an inner, or cytoplasmic, membrane, a rigid cell wall, and an outer membrane. All the components of the outer membrane are synthesized elsewhere in the cell: protein on ribosomes within the cell; lipid and lipopolysaccharide (LPS) in the inner membrane. Our goal is and was to define how these components are organized and assembled during outer membrane biogenesis.

Previous work in this laboratory suggested that newly synthesized LPS molecules are translocated from the inner membrane into discrete specialized regions of the outer membrane. This evidence was obtained by using a mutant of *E. coli* that forms LPS richer in carbohydrate when grown with galactose than when grown in its absence. The outer membrane is denser when it contains more carbohydrate than when it contains less, so fragments of outer membrane derived from cells grown in the presence of galactose can be separated by equilibrium centrifugation from fragments derived from cells grown in its absence. When cells were grown in the absence of galactose, galactose added, and membrane fragments prepared at various times thereafter, within 2-4 minutes (~ 10% of a doubling time), a small percent of the membrane fragments attained the very high density characteristic of fragments from galactose-grown cells. These suggested that they were points where the newly synthesized, dense LPS was inserted into the outer membrane.

These experiments were then extended to prove that these "insertion points" are truly intermediates in the addition of LPS to the entire outer membrane. When galactose was added to cells for 30 seconds and then removed by dilution, the dense mebrane fragments appeared, and then disappeared, suggesting that dense, new LPS is quickly dispersed throughout the remainder of the membrane. Control experiments indicated that the results did not come from artifacts of mixing or segregation of membrane fragments. Thin sections and negatively stained preparations of these "insertion points" were then examined in the electron miscrope. They consisted of apparently pure single membrane units in spheres of approximately 0.5-1% of the surface area of the original bacterium. From the fraction of membrane that appears in these fragments and the estimated percent yield, it can be calculated that there are 10 to 30 of these insertion points per cell.

Various mutants have been isolated from the strain used in the above experiment, including auxotrophs for various amino acids, and a strain blocked in the decarboxylation of diaminopimelic acid. These mutants will be used to specifically label protein and the peptidoglycan of cell wall, in order to determine the relationship of newly-synthesized cell wall to "insertion points" of the closely juxtaposed outer membrane, and the method of insertion of protein into the outer membrane.

The lipids of the outer membrane of the above-mentioned strain of *E. coli* have been analyzed and consist of approximately 90% phosphatidylethanolamine, 8% phosphatidylglycerol, and 1% cardiolipin, similar to published values for the outer membrane of *Salmonella*. The method of insertion of lipid into this membrane has been investigated by adding labeled serine to label newly-synthesized phosphatidylethanolamine, while adding galactose to permit synthesis of density-labeled lipopolysaccharide "insertion points." Preliminary experiments indicate that the highest concentration of newly synthesized lipid occurs specifically in those fragments that do *not* serve as insertion points for lipopolysaccharide. These results suggest that there may be separate distinct insertion points for lipid. Subsequent experiments will be necessary to test this hypothesis.

Significance to Biomedical Research and the Program of the Institute:

Study of the organization of the outer layer of the cell surface will contribute to understanding its structure and its interaction with the environment. Earlier work from this laboratory showed that the outer layer is the barrier to many charged molecules in coliform bacteria, including many drugs, and that this barrier can be reduced by exposing the cells to EDTA, thus making them drug-sensitive. The current studies are aimed at understanding the structural basis for this permability barrier, and such understanding may have implications for drug therapy.

Furthermore, the very heterogeneous membrane structure suggested by the preceding experiments, in which different areas are specialized for insertion of different molecules that then can move within the membrane to achieve their final location, appears similar to the situation in cells of higher organisms. Recent findings have suggested that for such cells, especially lymphocytes, association of specific membrane sites with specific molecules is followed by migration of these molecules elsewhere in and through the membrane. It is possible that study of the outer membrane of *E. coli*, which appears to have a relatively simple structure, may shed some light on mechanisms operative in the more complex membranes of higher organisms.

Proposed Course:

The specialized regions of lipopolysaccharide entry mentioned above will be isolated, and their composition further analyzed. The rate of diffusion of lipopolysaccharide from these points to the rest of the outer membrane will also be measured. Density-labeled lipid precursors will be used to try to density label those regions where newly-synthesized lipid enters the membrane. Mutants will be used to specifically label protein and cell wall peptidoglycan, in order to determine the relationship of these components to the specialized regions of outer membrane mentioned above.

Honors and Awards:

Dr. Loretta Leive has been appointed a member of the Editorial Board of *Bacteriological Reviews* for three years beginning January 1, 1973.

Publications:

Kulpa, C. F., Jr., and Leive, L.: Mode of insertion of lipopolysaccharide into the outer membrane of *Escherichia coli*. In Fox, C. F. (Ed.): *Membrane Research: First ICN-UCLA Symposium on Molecular Biology*. New York, New York, Academic Press, 1972, pp. 155-160.

Leive, L., and Davis, B. D.: The cell envelope; spores. In Davis, B. D., Dulbecco, R., Eisen, H. N., and Ginsburg, H. S.: *Microbiology*, 2nd edition. New York, New York, Harper and Row, 1973, pp. 105-146.

Leive, L., and Morrison, D. C.: Isolation of lipopolysaccharides from bacteria. In Ginsburg, V. (Ed.): *Methods in Enzymology - Complex Carbohydrates*, Vol. 28. New York, New York, Academic Press, 1972, pp. 254-262.

Serial No. NIAMDD-LBP-10
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Chemistry and Mechanism of Pyridoxal Phosphate Enzymes

Previous Serial Number: Same

Principal Investigator: Dr. Edith Wilson Miles

Other Investigator: Dr. Osao Adachi

Cooperating Units: None

Man Years:

Total:	2.2
Professional:	2.0
Other:	0.2

Project Description:

Objectives:

To gain further understanding of features of enzymes which contribute to their conformation, subunit interaction, and mechanism of action. Pyridoxal phosphate enzymes have been chosen for study because they are important in amino acid metabolism and because pyridoxal phosphate has optical and chemical properties which make it a useful probe of the active site of the enzymes to which it is bound.

Methods Employed:

Enzyme purification procedures were developed to prepare homogeneous B protein of tryptophan synthetase. Crystallization techniques were employed to grow crystals of B protein. Purified enzyme was characterized by disc gel and sodium dodecylsulfate gel electrophoresis, by kinetic studies, and by absorbance spectra.

Chemical modification and photooxidation studies were carried out on homogeneous B protein to determine the role of specific functional groups in the structure and function of the B protein. Optical studies were carried out on native and modified enzymes to determine changes in the environment of the cofactor and of aromatic residues and in the ability of modified enzyme to form enzyme-substrate intermediates. The optical methods used include absorbance, fluorescence, circular dichroism, and optical rotatory dispersion spectroscopy.

α-D-Serine was synthesized and characterized by nuclear magnetic resonance and mass spectroscopy. This substrate was used in stopped-flow and steady-state kinetic studies of the reactions of the B protein to determine the rate-limiting step in this reaction and the rates of intermediate steps. Kinetic studies were also conducted to determine the effects of specific modification on catalytic properties and to determine the pH dependency of the reactions of the B protein.

Major Findings:

The B protein has been purified and has been crystallized for the first time in a new purification procedure which gives a high yield of homogeneous enzyme. The catalytic properties of the new preparation have been compared with those of B protein purified by the standard procedure of Wilson and Crawford. The results indicate that the standard purification procedure, which contains heat steps at 56° and 80°, results in some alteration in the properties of the native B protein and that this alteration is avoided in the new, improved preparation.

The conversion of serine to pyruvate by the B protein alone has been found to be rate-limited by the removal of the α-proton of L-serine. Steady-state and stopped-flow kinetic studies with α-D-serine have identified the enzyme-substrate intermediate which accumulates before this rate-limiting step. The finding that the overall rate of pyruvate formation was dependent on an ionizing group with a pK of 6.7 indicated that a histidyl moiety might serve as a base-catalyst in the removal of the α-proton of serine. Further evidence for the essential role of a histidyl residue in the B protein has been provided by chemical modification studies with diethylpyrocarbonate and by photooxidation studies of the B protein in the presence of L-serine and pyrixodal-P.

Significance to Biochemical Research and the Program of the Institute:

These studies are clarifying the active site chemistry and mechanism of pyridoxal phosphate enzymes which are important in amino acid metabolism. They also give information on subunit interaction and enzyme regulation in multienzyme systems.

Proposed Course:

These studies will be continued and extended with long range goals of determining chemical and physical features of pyridoxal phosphate enzymes required for specificity, catalytic activity, maintenance of conformation, and protein-protein interaction.

Honors and Awards: None

Publications:

Miles, E. W.: Reaction of α^5-pyridoxal methyl chloride, an analog of pyridoxal 5'-phosphate, with the B protein of *Escherichia coli* tryptophan synthetase. *Biochemistry* 11: 4954-4961, 1972.

Miles, E. W., Fales, H. M., and Gin, J. B.: 5-(2-Chloroethyl)-3-hydroxy-2-methyl-4-pyridinecarboxaldehyde (α^5-pyridoxal methyl chloride) and its reaction with N^{α}-acetyl-L-lysine to form a new cyclic imino acid derivative of homopyridoxal. *Biochemistry* 1: 4945-4953, 1972.

Serial No. NIAMDD-LBP-11
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Molecular Basis of Biologic Specificity

Previous Serial Number: Same

Principal Investigator: Dr. Leonard D. Kohn

Other Investigators: Drs. Jere P. Segrest, Ramon L. Tate, and Syed M. Amir, and Mr. George Lee [Graduate Student from American University, whose work is accomplished as partial fulfillment of his degree requirements]

Cooperating Units: Dr. Roger J. Winand, Hopital de Baviere, Institut de Medicine, Liege, Belgium
Dr. Charles M. Lapiere, Department of Dermatology, Hopital de Baviere, Institut de Medecine, Liege, Belgium
Dr. Ralph J. Nossal, Physical Sciences Laboratory, Division of Computer Research and Technology, NIH
Dr. S. S. Kerwar, Roche Institute of Molecular Biology, Nutley, New Jersey
Dr. H. R. Kaback, Roche Institute of Molecular Biology, Nutley, New Jersey
Dr. Chaviva Isersky, Arthritis and Rheumatism Branch, NIAMDD
Dr. George R. Martin, Laboratory of Biochemistry, NIDR
Dr. E. Carwile LeRoy, Department of Medicine, Columbia College of Physicians and Surgeons, New York, New York
Dr. Jan Wolff, Clinical Endocrinology Branch, NIAMDD
Dr. John M. Utting, Department of Biology, Georgetown University, Washington, D.C.
Mr. George Poy, Arthritis and Rheumatism Branch, NIAMDD [on amino acid analyses]

Man Years:

Total:	4.6
Professional:	3.1
Other:	0.5

Project Description:

Objectives:

Analyses of the molecular bases for enzyme activity and specificity, the mechanisms by which such factors are regulated or determined, and the importance of these factors to such functions of the organism as hormone regulation and cellular transport. Studies of normal and abnormal hormonal factors, their production, their enzymatic expression, and their mechanism of action. Studies of membrane structure and function.

Methods Employed:

Procedures common to the purification, assay, and chemical modification of enzymes have been coupled with methods evaluating the size, shape, composition, and sequence of macromolecules.

Major Findings:

I. Relationships of Thyrotropin to Exophthalmogenic Factor and Studies of Thyrotropin Action on the Thyroid Gland and Retro-Orbital Tissues. Previous work demonstrated that TSH was exophthalmogenic but that partial pepsin digestion of TSH could yield an exophthalmogenic fragment of the TSH molecule which had lost its thyroid stimulating ability. This 20,000 molecular weight fragment consists of all of the β subunit and an amino terminal fragment of the α subunit of TSH. This fragment and TSH can cause exophthalmos in fish, guinea pigs, and mice.

Thyrotropin-induced exophthalmos in the guinea pig is associated with a two-fold increase in the glycosaminoglycan content of the predominant tissue of the retro-orbital space, the Harderian gland. Hyaluronic acid is the major glycosaminoglycan of the normal gland and accounts for over 90% of the two-fold increase. Sulfated glycosaminoglycans account for the remainder. Since the increase in sulfated glycosaminoglycans is proportional to the increase in hyaluronic acid, nondialyzable [^{35}S]sulfate incorporation into the Harderian gland is a sensitive index of these glycosaminoglycan increases. Dialyzable [^{35}S]sulfate incorporation measures an early effect of thyrotropin administration, *i.e.*, the conversion of inorganic sulfate to an activated sulfate derivative, phosphoadenosinephosphosulfate.

The glycosaminoglycan increases in exophthalmos can be induced by crude thyrotropin preparations, by purified and homogeneous preparations of thyrotropin, or by the 20,000 molecular weight proteolytic derivative of the thyrotropin molecule which has effectively no thyroid stimulating activity but is still exophthalmogenic in fish bioassays. These findings again indicate that thyrotropin is exophthalmogenic but that the determinant responsible for the exophthalmogenic activity is not the same as that responsible for thyroid stimulation. They suggest that the absence of thyroid stimulating activity in the serum of patients with exophthalmos does not exclude a role for thyrotropin in the pathogenesis of this disease.

Plasma membranes have been prepared from bovine thyroid cells and have been characterized as being reasonably free of other subcellular structures by electron microscopy and enzyme marker analysis. Bovine thyrotropin specifically binds to these membranes, and this binding can be correlated with adenylate cyclase activation. The binding of thyrotropin to the membranes is immediate, is inversely dependent on temperature, is inhibited by increasing salt concentrations, and is not affected by a range of mono-, di-, and trisaccharides. The thyroid-stimulating activity of the thyrotropin preparations is unaffected by incubation with membranes under conditions of the binding assay.

Both the α and the β subunits of bovine thyrotropin bind to thyroid membranes, β better than α on a molar basis; only the β subunit, however, inhibits thyrotropin binding at the concentrations tested. Mouse tumor thyrotropin is a potent inhibitor of bovine thyrotropin binding; human thyrotropin is not as effective. Since γ-globulin preparations with or without long acting thyroid-stimulating activity inhibit thyrotropin binding only slightly at high γ-globulin concentrations, the long acting thyroid stimulator itself appears to have no effect on thyrotropin binding to thyroid membranes. The TSH receptors of thyroid and retro-orbital tissues can thus be functionally distinguished, and additional credence is given to the possibility that alterations of normal hormone-receptor interactions can result in human disease.

Since one of the two subunits of luteinizing hormone (LH) has a primary amino acid sequence that is very nearly identical to the α subunit of TSH, we have examined the effect of LH on TSH binding and have tried to correlate this effect with several parameters of biological activity: adenylate cyclase activity of beef thyroid plasma membranes and the oxidation of [1-^{14}C]glucose by dog thyroid slices. These effects were, in turn, compared to those of the α and β subunits of TSH. The results show a high correlation between the binding parameter and these physiological responses; they emphasize the importance of chemical criteria rather than *in vivo* biologic potency as a measure of purity.

An antibody has been made to the exophthalmogenic fragment of the TSH molecule. Its cross reactivity with TSH and the subunits of TSH is under investigation.

(Drs. L. D. Kohn, S. M. Amir, R. L. Tate, J. Wolff [A:CE], and R. J. Winand [University of Liege, Liege, Belgium])

II. Procollagen and Procollagen Peptidase. Procollagen peptidase from bovine tendons has been purified. The final preparations contain two active components, molecular weights ~ 70,000 and ~ 140,000. Both active species are precipitated by an antibody to the enzyme and by immunoelectrophoretic analysis have common antigenic components. Since rechromatography of any one component always yields both components, the enzyme is presumed to be a polymerizing system composed of 70,000 molecular weight monomers. Both forms work on both pro α1 and pro α2 chains and are EDTA inhibited. The 70,000 molecular weight species is not Ca^{2+} reactivated, whereas the 130,000 molecular weight species is. They prefer native procollagen (K_m 10^{-6} M) as substrate as opposed to the individual pro α1 and pro α2 chains (K_m 10^{-3} M). The enzyme is an endopeptidase which cleaves the amino terminal peptide en bloc. The amino acid composition and molecular weight of the excised peptide have been determined.

Procollagen synthesis has been studied in L929 cells, a fibroblast culture of a mouse tumor. These cells are analogous to dermatosparaxic in their low level of procollagen peptidase activity in the media, and approximately 25% of their ribosomal product is procollagen. The cells contain precursor chain aggregates which are disulfide bonded, *i.e.*, the procollagen molecule forms intracellularly and appears to be disulfide bond stabilized.

Three patients with a variant of the Ehlers-Danlos syndrome, a generalized disorder of connective tissue, have detectable amounts of procollagen in extracts of their skin and tendon. The activity of procollagen peptidase, the enzyme which converts procollagen to collagen, was reduced in their skin fibroblast cultures. The clinical manifestations of this syndrome may be related to impaired enzymatic conversion of procollagen to collagen. Skin fibroblast cultures from these patients have an increased synthetic rate of collagenous (collagen and procollagen) protein, possibly related to the inability to convert procollagen to collagen.

(Drs. L. D. Kohn, C. Isersky [A:A&R], C. M. Lapiere [Hopital de Baviere, Liege, Belgium], S. S. Kerwar [Roche Institute of Molecular Biology, Nutley, New Jersey], G. R. Martin [D:LB], and E. C. LeRoy [Columbia University College of Physicians and Surgeons, New York City], Mr. G. Lee [American University], and Mr. G. Poy [A:A&R])

III. Membrane Transport. The membrane-bound D-lactate dehydrogenase of *Escherichia coli* ML 308-225 has been solubilized and purified to homogeneity. The enzyme has a molecular weight of 75,000 ± 7% and contains approximately 1 mole of flavin adenine dinucleotide per mole of enzyme. Its pH optimum before exposure to Triton X-100 is 7.5 to 8.0; after exposure it is 8.0 to 9.5. The purified enzyme has a K_m value of 1.4×10^{-3} M for D-lactate and 3.0×10^{-2} M for L-lactate before exposure to Triton X-100; after exposure its K_m values are 0.5×10^{-3} M and 2.1×10^{-2} M for the same substrates, respectively. The enzyme is not active with D-glycerate, L-glycerate, succinate, malate, D-tartrate, L-tartrate, mesotatrate, n-propanol, or isopropanol as substrates and oxidized diphosphopyridine nucleotide has no effect on the catalytic conversion of D-lactate to pyruvate. The enzyme is not inhibited by p-chloromercuribenzoate, N-ethylmaleimide, or 5,5'-dithiobis(2-nitrobenzoic acid), but is inactivated by treatment with 2-hydroxy-3-butynoic acid.

Recent experiments demonstrate that guanidine extracts from wild type vesicles containing D-lactate dehydrogenase activity are able to reconstitute D-lactate-dependent oxygen consumption and active transport in membrane vesicles from an *E. coli* mutant deficient in D-lactate dehydrogenase.

(Drs. L. D. Kohn and H. R. Kaback [Roche Institute of Molecular Biology, Nutley, New Jersey])

IV. Membrane Structure. The membrane-penetrating MN-glycoprotein of the human erythrocyte has been examined by chemical and physical techniques. The intramembranous portion of this glycoprotein is shown to belong to an unusually hydrophobic class of peptides which includes the membrane spanning antibiotic, gramicidin A. Circular dichroism data show that this intramembranous region forms an unusually stable helix in hydrophobic environments, and in the absence of strong perturbations should be helical *in situ*. The length of this helix (35 Å) is of the order of the thickness of the hydrophobic core of the membrane. The intramembranous peptide has a strong affinity for cholesterol and phospholipids and contains covalently bound phosphorous which is not associated with phospholipid.

(Drs. J. P. Segrest and L. D. Kohn)

V. Structure-Function Studies of Hydroxypyruvate Reductases. The hydroxypyruvate reductase induced in *P. acidovorans* by growth on glyoxylate has a molecular weight of approximately 80,000. From sodium dodecylsulfate gels and ultracentrifuge studies a subunit molecular weight of 40,000 has been calculated for the induced enzyme. The subunits of the induced enzyme are identical and can reassociate to yield a protein having the same physical and kinetic properties as native enzyme. The enzyme has four half-cystine residues, all of which act as free sulfhydryl residues and are important to activity. A single peptide containing two sulfhydryl residues has been isolated from peptide maps of the digested subunit preparations. Substrate protection studies suggest the sulfhydryl residues are at the enzyme active site.

The enzyme binds two moles of DPNH per mole of native enzyme. It has an ordered reaction mechanism in which DPNH binds before the carbonyl substrate.

(Drs. L. D. Kohn and J. M. Utting [Georgetown University])

VI. Bacterial Chemotaxis. Studies have been initiated to determine force-response relationships pertaining to chemotactic motions of *E. coli* K_{12} bacteria. Assay techniques for bacterial motion utilize newly developed methods of laser light intensity correlation spectroscopy. The isolation of chemotactic receptors is also being attempted, with subsequent analysis of receptor function when embedded in reconstituted lipid bilayer membranes.

(Drs. R. J. Nossal [CR:PSL] and L. D. Kohn)

Significance to Biomedical Research and the Program of the Institute:

Analyses of the molecular bases for activity and specificity and the mechanisms by which these determinants are influenced offer a further understanding of the nature of metabolic and endocrine defects. The possibility that unregulated biosynthetic or degradative processes may convert TSH into EPS active material has been established, and a mechanism for human exophthalmos has been proposed on the bases of these data. Preliminary studies of procollagen, procollagen peptidase, and collagen biosynthesis suggest an important relationship to aging and to the development of connective tissue diseases.

Proposed Course:

Structure-function analyses of the reductase model system are being pursued. The localization of the active and regulatory sites on each enzyme will be sought and the comparative structures of these units will be compared. Comparison of membrane bound and free enzymes will be made. Similar studies of the TSH-exophthalmic factor relationship will be continued in an attempt to examine the mechanism by which EPS activity is generated *in vitro* and *in vivo*. The proposed mechanism for human exophthalmos will be tested. The mechanism of procollagen peptidase formation and control will be related to normal and disease states.

Honors and Awards: None

Publications:

Amir, S. M., Carraway, T. F., Kohn, L. D., and Winand, R. J.: The binding of thyrotropin to isolated bovine-thyroid plasma membranes. *J. Biol. Chem.*, in press (June 1973).

Kerwar, S. S., Cardinale, G. J., Spears, C. L., Stassen, F. L. H., and Kohn, L. D.: Studies on the cell-free synthesis of procollagen by L929 fibroblasts. *Proc. Nat. Acad. Sci. U.S.A.* 70, in press (May 1973).

Kerwar, S. S., Kohn, L. D., Lapiere, C. M., and Weissbach H.: *In vitro* synthesis of procollagen on polysomes. *Proc. Nat. Acad. Sci. U.S.A.* 69: 2727-2731, 1972.

Kohn, L. D., and Winand, R. J.: Studies of experimental exophthalmos: a proposed mechanism for the pathogenesis of the human disease. *Israel J. Med. Sci.*, in press.

Nossal, R. J.: The growth and movement of rings of chemotactic bacteria. *Exp. Cell Res.* 75: 138-142, 1972.

Nossal, R. J., and Chen, S. H.: Effects of chemoattractants on the motility of *Escherichia coli*. *Nature*, in press.

Segrest, J. P., and Kohn, L. D.: Protein-lipid interactions of the membrane-penetrating MN-glycoprotein from the human erythrocyte. *Protides of the Biological Fluids*, 21st Annual Colloquium on Protides of the Biological Fluids, Brugge, Belgium, 1973, in press.

Winand, R. J., and Kohn, L. D.: Retrobulbar modifications in experimental exophthalmos: the effect of thyrotropin and an exophthalmos producing substance derived from thyrotropin on the $^{35}SO_4$ incorporation and glycosaminoglycan content of Harderian glands. *Endocrinology*, in press (September 1973).

Winand, R. J., and Kohn, L. D.: The binding of [^{3}H]thyrotropin and an [^{3}H]-labeled exophthalmogenic factor by plasma membranes of retro-orbital tissues. *Proc. Nat. Acad. Sci. U.S.A.* 69: 1711-1715, 1972.

Winand, R. J., and Kohn, L. D.: The pathogenesis of human and experimental exophthalmos. *Proceedings of the International Endocrine Congress*, Washington, D.C., 1972. *Excerpta Medica International Congress Series*, in press.

Serial No. NIAMDD-LBP-12
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Role of Subunit Interactions in Enzyme Chemistry

Previous Serial Number: Same

Principal Investigator: Dr. Claude B. Klee

Other Investigators: None

Cooperating Units: Drs. Werner Klee and K. Subramonia Iyer, Laboratory of General and Comparative Biochemistry, National Institute of Mental Health
Drs. Louis A. Cohen, Kenneth L. Kirk, and Herman J. C. Yeh, Laboratory of Chemistry, NIAMDD
Dr. Peter McPhie, Laboratory of Biochemistry and Metabolism, NIAMDD

Man Years:

Total:	1.7
Professional:	1.0
Others:	0.7

Project Description:

Objectives:

Two enzymatic systems, histidine ammonia-lyase and lactose synthetase, are used to study the possible regulatory functions of protein-protein interactions.

Methods Employed:

The project involves the study of properties of proteins, purified by the usual techniques of protein isolation, including many types of chromatography. The enzymes are being studied by examining their optical, hydrodynamic, and kinetic properties as well as by suitable chemical measurements and modifications.

Major Findings:

I. Histidine Ammonia-Lyase. Histidine ammonia-lyase catalyzes an α,β elimination of NH_3 from histidine to produce urocanic acid. This reaction was shown to be stimulated by metal ions and previous work in this laboratory has indicated that each of the 4 subunits of the tetrameric enzyme contributes one reactive cysteinyl residue to the metal binding site of the enzyme. The active center of the enzyme has been shown to contain an electrophilic center which interacts with the α amino group of L-histidine and is therefore directly involved in the catalytic process. Our present studies are directed toward the elucidation of the exact nature and the stoichiometry of the active center and its relationship to the metal binding site in order to understand the mechanism of this elimination reaction.

II. Active Site Structural Studies. The electrophilic center of the enzyme contains a novel prosthetic group believed to be a dehydroalanine-imine system. Treatment of the enzyme with sodium sulfite at pH 7.0 results in inactivation and formation of cysteic acid. Thus, the pre-existence of the dehydroalanine residues at neutral pH is demonstrated. Prior studies with $NaBH_4$ were performed at high pH and it was conceivable that dehydroalanine formed only under these conditions. Furthermore, the modified residue is a very stable and easily characterized compound which makes this technique a rapid and very sensitive one with which to determine the existence of unsaturated amino acids in proteins. The stereospecific inhibition of the enzyme by L-cysteine has been characterized further with the hope that it will enable us to determine the structure of the still unknown residue which interacts with the amino group of dehydroalanine. Since the enzyme inactivated by L-cysteine fails to incorporate ^3H into alanine upon treatment with $[^3H]NaBH_4$, it is believed that both reagents act at the same locus. The reaction of the enzyme with L-cysteine occurs in 2 steps: a fast inactivation, which corresponds to the incorporation of 3-4 moles of $[^{14}C]$- or $[^{35}S]$cysteine per mole of enzyme and requires a high pH, and a low modification which can be monitored by changes in the UV absorption at 340 nm. This slow modification also requires high pH and is dependent upon the presence of the bound cysteine. The nature of the linkage between cysteine and the enzyme is not clearly understood yet, but does not seem to involve the formation of a disulfide bridge or an addition accross the double bond of dehydroalanine. Pronase treatment of the cysteine modified enzyme yields a 20-30 residue peptide which contains the radioactive cysteine and also has the characteristic UV absorption peak at 340 nm. We hope that the elucidation of the structure of this peptide will enable us to elucidate the nature of the residue which interacts with L-cysteine and to determine the number of active centers which is still the subject of some uncertainty.

III. Mechanism of Action. We have pursued our investigation of the mechanism of the reaction catalyzed by the enzyme using the substrate analog 4-fluoro-L-histidine. The reaction was shown to be reversible and an equilibrium constant of 5.0 was determined for both fluorohistidine and histidine. Transient state kinetics clearly show that the rate determining step in the deamination of 4-fluoro-L-histidine precedes the dissociation of the amino enzyme in the following reaction sequence:

$$\text{fluorohistidine} + \text{enzyme} \rightleftarrows \text{fluorourocanic acid} + H^+ + \text{amino enzyme}$$

$$\text{amino enzyme} + H^+ \rightleftarrows \text{enzyme} + NH_3.$$

The existence of a deuterium isotope effect with 4-fluoro-(βD_2)-histidine and the absence of a detectable fluorourocanate exchange with fluorohistidine indicates that the breakage of the C-H bond participates in the rate determining step. Further studies with T_2O exchange should help to decide if the proton liberation or a concerted C-H, C-N bond cleavage is rate determining. Since these results are in contrast to the postulated rate determining step in the deamination of histidine, which is believed to be the dissociation of the amino enzyme, it is possible that substitution of the H with F in the 4 position of the imidazole ring results in a greatly reduced rate of covalent bond cleavage and therefore a change in the rate determining step.

(Drs. C. B. Klee, L. A. Cohen (A:LC], K. L. Kirk [A:LC], and P. McPhie [A:LBM])

IV. Nuclear Magnetic Studies. Nuclear Magnetic studies of the interactions of the enzyme metal complex with Cl^- ions and the effect of the substrate L-histidine or fluorohistidine on these interactions will be carried out in order to decide if the metal ions have a direct or indirect role in the enzyme catalysis.

(Drs. H. J. C. Yeh [A:LC], C. B. Klee, L. A. Cohen [A:LC], and K. L. Kirk [A:LC])

V. Lactose Synthetase. α-Lactalbumin, one of the subunits of the lactose synthetase enzyme complex has been guanidinated by treatment with O-methylisourea. The guanidination reaction proceeds smoothly and all 12 lysine residues may be converted to homoarginine without sensible change in either the structure of the protein or its activity in specifying lactose synthesis. With the use of radioactively labeled reagent, 12 moles of ^{14}C have been introduced per mole of α-lactalbumin in stable covalent linkage. This material should prove to be useful for binding studies with subcellular fragments containing galactosyl transferase activity. A side reaction noticed for the first time in these studies is the hydrolytic cleavage of disulfide bonds followed by disulfide interchange leading to polymer formation during the guanidination procedure.

(Drs. W. Klee [M:LGCB], K. S. Iyer [M:LGCB], and C. B. Klee)

Significance to Biomedical Research and the Program of the Institute:

Histidine ammonia-lyase and phenylalanine ammonia-lyase are the only enzymes so far known to have unsaturated amino acid residues as part of their active sites. Doubtless, other such enzymes will ultimately be described and an understanding of the mechanism of action of the one protein will be of use in understanding the mode of action of the whole class. The mechanisms by which such unsaturated amino acids arise, both in proteins and in some peptide antibiotics, are open questions but may well have implications for the structure of some cross-linked proteins. Dehydroamino acids formed as a result of the elimination of various groups on the β carbon of amino acid residues may be important in structural as well as functional roles.

Proposed Course:

Future work with histidase will continue to be aimed at the elucidation of the structure of the active site which will enable us to understand the mechanism of action of this enzyme. Ultimately, we hope to understand the participation of each of the 4 subunits in the formation and function of the active center. As part of our work on protein-protein interaction, we hope to use the modification of α-lactalbumin, the "specifier" protein of the lactose synthetase complex as a tool to study its interactions with the catalytic component UDP-galactosyl transferase.

Honors and Awards: None

Publications:

Klee, C. B., and Gladner, J. A.: Isolation of a cysteine-peptide at the active site of histidine ammonia-lyase. *J. Biol. Chem.* 247: 8051-8057, 1972.

Klee, W. A., and Klee, C. B.: The interaction of α-lactalbumin and the A protein of lactose synthetase. *J. Biol. Chem.* 247: 2336-2344, 1972.

LABORATORY OF CHEMICAL PHYSICS

Organization: The Laboratory of Chemical Physics was established in June 1972 from units of the Laboratory of Physical Biology. Two new sections--Spectroscopy and Molecular Structure, and Membrane Biophysics--were formed to supplement the three existing sections--Physiology, Molecular Biophysics, and Electrochemistry and Colloid Physics. The last named section was abolished following the recent retirement of Dr. Karl Sollner after a long and distinguished career in Europe and the U.S., the last 26 years at NIH. Dr. Sollner remains active in research as a consultant to NIAMDD.

Summary of research: A wide range of spectroscopic techniques is being employed to investigate in detail the electronic states, chemical bonding and structure of molecules.

In the small molecule category, Kon, has found by electron paramagnetic resonance that the torsional oscillation of oxygen is not prevented when the oxygen is dissolved in solid carbon monoxide and nitrogen matrices nor is the structure of the valence electrons perturbed from that of the gaseous state in the non-polar nitrogen matrix. In collaboration with Szent-Gyorgyi of the Woods Hole Marine Biological Laboratories he has also confirmed by electron paramagnetic resonance that the transient visible absorption which occurs during Schiff base formation is a charge transfer transition and that the transition is accelerated by minute quantities of sulfur compounds such as glutathione.

McDiarmid has assigned the vacuum ultraviolet transitions in tungsten, molybdenum and rhenium hexafluorides to the promotion of a fluorine valence electron to an unfilled degenerate orbital of the metal. In the sulfur analogue of ether she has found several transitions of a relatively rare type consisting of the promotion of an electron from an orbital lower in energy than that of the highest filled orbital. Photochemical reactivity from states reached by this promotion could be substantially different from normal.

Turning to the spectroscopic studies of large molecules, Eaton and Hofrichter have been examining sickled erythrocytes containing homozygous hemoglobin S and have found that the long axis of the hemoglobin molecule is oriented within 20° of the long axis of the microtubules found in the sickled erythrocytes. They conclude from this that the hemoglobin S mutation sites are not involved in the longitudinal contacts but are involved in the lateral aggregation within microtubules or between microtubules. Eaton (with Makinen, formerly NIDR-LB) has also investigated the optical spectrum of single crystals and the circular dichroism of solutions of a variety of heme protein derivatives; extensively characterized their excited states; and found, among other things, supporting evidence for the Hoard-Perutz hypothesis that the iron-porphyrin displacement is the sterochemical trigger for cooperative oxygen binding in hemoglobin.

In another heme protein study, Kon and Nebert (NICHD-LBS) have shown that a characteristic transition to an Fe(III) state induced in cytochrome P-450 in vivo by aromatic hydrocarbons and phenobarbital injection occurs only in genetically responsive animals. Still in porphyrin studies, Olson has succeeded in obtaining very thin sections of large bacteriochlorophyll-protein crystals and is currently examining their polarized spectra.

Other large molecule research is being done by Charney on polynucleotides, DNA and polypeptides using the technique of orienting these molecules by means of pulsed electric fields. The temperature dependence of these measurements with low molecular weight DNA has shown that the molecule is anistropically polarizable but has a relatively small permanent dipole moment. Charney and Hofrichter also have a study under way of the dichroism of fibres of DNA. This research has as its object a determination of the optical anisotropy of the DNA molecules for comparison with observations made on DNA in solution by electric dichroism. In another study with Kohn (NCI) and Glaubiger (formerly NCI) the complex of the antibiotic anthramycin with DNA has been shown to bind very differently from that of most other planar molecules whose complexes have been examined. The latter bind by intercalating between the DNA base pairs and parallel to them while anthramycin makes an angle of about 40° with the nucleoside bases.

From an analysis of infrared and Raman spectra of a small molecule (especially one possessing some symmetry) it is in principle possible to determine the force constants responsible for the vibrations of the molecule. Levin and Pearce have been investigating the extent to which such force constants can be accurately determined in practice and can then be utilized to predict or interpret the vibrational spectra of more complex molecules. They have successfully analyzed in detail the vibrational spectra of a series of related molecules: azomethane ($CH_3-N=N-CH_3$), cis- and trans-1,2-difluorodiazine ($F-N=N-F$), cis- and trans-1,2-difluoroethylene ($F-CH=CH-F$), and cis- and trans-2-butene and 2-butene-d_8 ($CH_3-CH=CH-CH_3$).

NMR spectroscopy often provides a detailed picture of the chemical structure and conformation of molecules and of the way in which different molecules interact with each other. For example, Gupta has shown by NMR that cytochrome-c, an electron transfer hemoprotein, interacts reversibly with cytochrome-c-peroxidase, a heme-containing enzyme, to form a 1:1 complex, with no evidence for heme-heme interaction in the complex. Tucker and Becker used NMR, as well as infrared spectroscopy and high precision vapor pressure determinations, to establish quantitatively the species present in the self association of t-butanol by hydrogen bonding. This work clears up several long-standing conflicts and provides a basis for understanding hydrogen bonding in many other alcohols and phenols.

Wasylishen has used molecular orbital theory at the INDO level of approximation to calculate nuclear spin-spin coupling constants in ammonia as a function of the HNH angle. Preliminary experimental measurements on several ^{15}N-anilines indicate that the observed spin-spin coupling constants may be used together with these calculations to estimate the planarity of the NH_2 fragment.

A major project of this Laboratory during the last few years has been the development of methods for rapidly obtaining NMR spectra, primarily in order to employ time-averaging procedures to enhance signal/noise. Bradley and

Pitner (in collaboration with personnel of DCRT and BEIB) have made substantial progress in the development of a high magnetic field pulsed spectrometer for ^{13}C NMR, while Gupta (with Ferretti, DCRT) has explored both theoretically and experimentally the advantages of a new NMR method, correlation spectroscopy. Pitner and Becker have designed apparatus for and have initiated studies of rapid reactions by NMR with the use of pulse and Fourier transform methods.

Two serious errors in molecular structure in the chemical literature were discovered in LCP during the year. Weiss and Rice, in the course of attempting to prepare a specific nitroxyl free radical for use as a spin label, found that the reaction product of phorone and hydroxylamine, long thought to be N-hydroxy-2,2,6,6-tetramethyl-4-piperidone, is actually hexahydro-3,3,7,7-tetramethyl-1,2-oxazepin-5-one, a seven-membered ring compound whose synthesis had previously been thought possible only by rather involved methods. (This oxazepin is conformationally mobile; Wasylishen and Rice have found by NMR a barrier to inversion of 19 kcal/mole, approximately independent of the substituent on the nitrogen atom.) Ziffer and Seeman showed that the stereochemistry previously assigned by other workers to the major and minor photoproducts of A-homo-cholestan-3-one was reversed. This stereochemistry is critical in understanding the singlet and triplet photochemistry of β,γ-unsaturated ketones. These two photoproducts have also been shown to undergo stereospecific oxa-di-π-methane rearrangement on acetone-sensitized irradiation.

Shean and Sollner are studying the electrochemistry of liquid cation exchanger membranes, with particular emphasis on membranes incorporating the strong acid dipicrylamine (DPA). Apparent anomalies in electrical cell potentials were found to be due to solubility of DPA in the aqueous phases and resultant reduction in the nominal concentration ratios of the salts in the cells.

The physicochemical mechanism of light detection by the retina has continued to be investigated in several different ways. Adams, Jennings and Sharpless have used retinaldehyde, the chromophoric group of animal vision, to synthesize model compounds (Schiff bases) with primary amino groups of amino acids, peptides, and phospholipids, the aim being to make compounds of known structure with the unusual spectral absorption properties of rhodopsin, the pigment of rod vision. Yoshikami, Penn and W. Hagins analyzed the electric currents of vertebrate rods and showed that they involve a continuous inflow of Na^+ in darkness into rod outer segments. The current is interrupted when light is absorbed in internal rhodopsin-bearing discs in the outer segments. Four different types of experiments now point to the existence of an excitatory mechanism in which the rod disks release Ca^{++} ions on illumination and these diffuse to the plasma membrane where they transiently reduce its Na^+ permeability.

F. Hagins analyzed the protein component of purified squid rhodopsin and found that it consists of a single peptide chain of MW 50 K Dalters. It is rich in non-polar amino acids and has an N terminal glycine.

Experiments by Altland and Sellner (with Highman, formerly LEP, NIAMDD) on the effects of cross-adaptation between cold and altitude (hypoxia) have shown that cold-acclimated rats exhibit poor hypoxic tolerance when exposed to 6.6 or 4.9% O_2 in the cold (1.7°C). The cold-acclimated rats showed greater increases than unacclimated rats in oxidative and glycolytic serum enzymes, higher incidence of renal tubular dilatation, fatty changes in striated muscles, and severe hepatic glycogen depletion and maintained higher body temperatures during exposure. The reduced tolerance is attributed to an increased metabolism and severe tissue hypoxia.

Altland previously reported that exposure to altitude, cold and exercise increased the susceptibility of rats to experimental bacterial endocarditis. Physical training has been found to reduce the incidence and severity of the endocarditis in exercised epinephrine-treated rats, apparently as a result of an improved cardiac capillary density and circulatory level which facilitated the timely passage of leucocytes and other humoral defensive factors to and removal of toxic agents from the endocardium and valvular leaflets.

Serial No. NIAMDD-LCP-1
1. Chemical Physics
2. Electrochemistry and Colloid Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The physical chemistry of membranes and complex membrane systems of biological interest

Previous Serial Number: NIAMD-LPB-6

Principal Investigator: Karl Sollner (retired January 31, 1973)

Other Investigators: Gerald M. Shean

Cooperating Units: None

Man Years:

Total:	2
Professional:	1.5
Other:	.5

Project Description:

Objectives:

The physico-chemical study of membranes, and complex membrane systems and models with the ultimate aim of providing a rational basis for various complex membrane phenomena in living systems.

Methods Employed:

Conventional chemical-analytical and electrochemical methods, especially potentiometric measurements as reported previously.

Major Findings:

The principal investigator retired as of January 31, 1973, and has worked up to that date more or less exclusively on a historical survey of the physical chemistry of membranes (and continued to do so after retirement); there are no "Major Findings."

The results of the experimental work carried out by Dr. Shean can be summarized as follows: Studies on the electrochemistry of liquid cation exchanger membranes were further pursued with particular emphasis on membranes incorporating the strong acid dipicrylamine (DPA), 2,2',4,4',6,6' hexanitrodiphenylamine. With both concentration potentials and bi-ionic potentials (BIPs) the measured potentials were less with dilute electrolyte solutions, e.g. nominally .001 N, than with higher concentrations, and is more pronounced the lower the cation is in the Hofmeister series. This was not due to any leakage of anions through the membranes, but was due to the solubility of the DPA in the aqueous phases, which reduced the nominal 2:1 ratio of concentration cells and the nominal 1:1 ratio of BIP cells. When the true cation ratios were taken into account, the measured concentration potentials closely approach the calculated and the BIPs are essentially independent of concentration as predicted by theory. The measurement of concentration potentials with nominally 0.2 N MCl vs. 0.1 N MCl with a variety of exchangeable cations showed that all of the common inorganic univalent cations, with the exception of lithium, gave, at this concentration level, potentials very close to those predicted by theory. The less than theoretical potential for lithium was due to the solubility of the LiDPA in the aqueous phases which decreased the nominally 2:1 ratio of lithium. Using the same variety of cations in bi-ionic cells, e.g. DPA in o-nitrotoluene membranes separating .1 N chloride solutions, BIPs and the transference ratios of the cations were obtained. Those cations higher in the Hofmeister series, as expected, were able to impress their positive charge on the other solution at opposite side of the membrane.

The following values (signs indicating the potassium side) were found for cations relative to potassium:

			BIP	$\frac{\text{Transference Cation}}{\text{Transference Potassium}}$
0.1 N KCl	vs.	0.1 N Me_4NCl	+198.8mv.	2,320.0
"	"	0.1 N CsCl	+ 76.0mv.	19.3
"	"	0.1 N RbCl	+ 39.1mv.	4.6
"	"	0.1 N KCl	0mv.	1.0
				$\frac{\text{Transference Potassium}}{\text{Transference Cation}}$
"	"	0.1 N NH_4Cl	- 31.8mv.	3.45
"	"	0.1 N NaCl	-108.2mv.	67.70
"	"	0.1 N $CaCl_2$	-129.7mv.	157.00
"	"	0.1 N LiCl	-148.5mv.	324.00

When BIPs were measured with cells that did not contain potassium, the potentials were the same as those calculated from data found with cells containing potassium, i.e. the BIPs were algebraically additive. For example: 0.1 N RbCl vs. 0.1 N NH_4Cl measured 70.4mv (NH_4 side positive) while the calculated was 39.1mv. + 31.8mv. = 70.9mv.

Significance to Biomedical Research and the Program of the Institute:

Movement of solvent and solutes is an important aspect of all life processes. This project tries to provide solidly founded, nonspeculative physicochemical background information on the functional behavior of membranes in general. At the same time, these membrane studies have furnished a new, now widely used tool for the electrometric determination of ion activities - the use of permselective membranes as membrane electrodes. It is perhaps also of interest that Sollner's "permselective" membranes are the prototypes of the ion exchange membranes which are used now in the electrodialytic desalination of sea and brackish water.

Proposed Course of the Project:

Continuation of the systematic work on cation anion and exchanger membranes, particularly polyionic potentials. Continuation of the search for more desirable ion exchanger compounds. Specifically, studying with liquid ion exchanger membranes the effect on concentration potentials and BIPs of hydrogen-bonding solvents as compared to solvents that are electron donors. Investigating a variety of non-nitrogen containing anion exchangers for their use in liquid membrane studies.

Publications:

Shean, G. and Sollner, K.: Bi-ionic potentials across liquid anion exchanger membranes. *J. Membrane Biol.* 9: 297-304, 1972.

Serial No. NIAMDD-LCP-2
1. Chemical Physics
2. Membrane Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The physical and chemical bases of photoreception

Previous Serial Number: NIAMD-LPB-11

Principal Investigator: W. A. Hagins

Other Investigators: W. H. Cobbs, III
W. E. Robinson

Cooperating Units: Frances M. Hagins (Guest Worker, LCP-NIAMDD)
Shuko Yoshikami (Guest Worker, LCP-NIAMDD and NEI-LVR)
Hartmann Rüppel (Max Volmer Institute, Berlin, Germany)

Man Years

Total:	3.5
Professional:	3.5
Other:	0

Project Description:

Objectives:

To outline the successive events by which light quanta absorbed in the receptors of animals lead to the transmission of information to the brain.

Methods Employed:

1. Measurements of currents, voltages, and impedances of the retina by conventional electronic methods.
2. Measurement of photochemical changes in rhodopsin in living cells by kinetic microspectrophotometry.
3. Analysis by digital computation of electrical signals produced by photoreceptors.
4. Investigation of the chemical structure of rhodopsin by spectrophotometry and amino acid analysis.
5. Electron microscopy of the cellular structure of photoreceptors.
6. Analysis of the ionic composition of the retina by electron probe microanalysis.
7. Study of the enzyme activity and ionic fluxes in isolated photoreceptive organelles.

Major Finds:

1. The investigation of the possible role of calcium as an intracellular sensory transmitter in rod outer segments has continued. The plasma membranes of rat rod outer segments have been made permeable to Ca^{++} by treatment with an ionophoric antibiotic, X 537A. When this is done, the normal dark current of rods is suppressed except at very low external Ca^{++} concentrations. From this it can be deduced that the current-suppressing effect of Ca^{++} in the fluid around retinal rods is caused by Ca^{++} ions which leak through the plasma membrane and affect its permeability to Na^+ from the inside. This is exactly what would be expected if Ca^{++} were a sensory transmitter released by light from the retinal rod disks. (Yoshikami and W. A. Hagins)

2. The fast photoelectric charge displacement (FPV) of rat rods has been analyzed into two components, one called the R2 wave and discovered by other laboratories and one, "the L wave," which was first identified by our group. Conditions have been found for separately determining the shape of each component and for analyzing mixtures of the two waveforms into its components. The L wave shows properties that are consistent with its being caused by movement of Na^+ and Ca^{++} ions through the plasma membrane via light created ionophoric sites in the rhodopsin molecules themselves. Separate studies indicate that a single absorbed photon causes the release of at least 1000 calcium ions into a rod outer segment. (Yoshikami and W. A. Hagins)

3. Analytical gel electrophoresis studies have shown that the rhodopsin of squid rod outer segments constitutes the most numerous of all the peptides present in the light-sensitive ends of the photoreceptor cells. (F. Hagins)

Significance to Biomedical Research and the Program of the Institute:

Progress in the understanding of molecular events underlying excitation of photoreceptors is obviously important in research on vision. In addition, it may provide a point of attack on the nature of permeability changes which occur in the surface membranes of electrically active cells, such as nerve and muscle, during transmission of nerve impulses and initiation of muscular contraction.

Proposed Course of Project:

In the immediate future three kinds of experiments are to be carried out:

1. Direct intracellular injection of Ca^{++} and Ca^{++} buffers into lining vertebrate rods. These experiments will provide crucial tests of the Ca^{++} hypothesis of excitation.
2. Studies of the ionic fluxes of rod outer segments which have been freshly isolated after labeling with Ca^{++}, Na^+ and K^+ will be done to estimate ionic turnover rates associated with the light response.

3. Attempts are being made to get a complete inventory of the peptides in single rod outer segments. The aim is to account biochemically for the ionic accumulation and release mechanisms which biophysical experiments indicate to be present.

Publications:

Rüppel, H., and Hagins, W. A.: Spatial origin of the fast photovoltage in retinal rods. Symp. Biochem. Visual Pigm. (in press).

Yoshikami, S., and Hagins, W. A.: Control of the dark current in vertebrate rods and cones. Symp. Biochem. Visual Pigm. (in press).

Hagins, F. M.: Purification and partial characterization of the protein component of squid rhodopsin. J. Biol. Chem. (in press).

REPORTED IN PRESS - 1972

Penn, R. D., and Hagins, W. A.: Kinetics of the photocurrent of retinal rods. Biophys. J. 12: 1073-1094, 1972.

Hagins, W. A.: The visual process: Excitatory mechanisms in the primary receptor cells. Annu. Rev. Biophys. Bioeng. 1: 131-158, 1972.

Serial No. NIAMDD-LCP-3
1. Chemical Physics
2. Membrane Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Digital computer facilities for LCP and LMB

Previous Serial Number: NONE

Principal Investigator: William H. Jennings, Jr.

Other Investigators: NONE

Cooperating Units: Computer Systems Laboratory, DCRT

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

Development of time-sharing computer facilities for on-line computation of data from various scientific instruments in Building 2.

Methods Employed:

Computer software development and testing; interfacing laboratory apparatus with the time-sharing computer.

Major Findings:

During fiscal 1973, the Building 2 computer system (Honeywell 516) came into wider use than ever before. New laboratory instruments were connected to it and some of its peripheral equipment that had formerly been leased was purchased in order to reduce future expenses. To date seven scientific papers, listed below, have appeared reporting experiments conducted or data processed with the aid of the system. The following laboratory instruments and other computers are now connected directly to this computer:

Cary 118	Ultra-violet spectrophotometer
Cary 60	Optical Rotatory Dispersion spectrometer
Beckman IR-7	Infra-red spectrophotometer

Cary 14	Spectrophotometer
Varian C-1024	Signal averaging computer
Raytheon 704	Mini computer
PDP-8	Mini computer
IBM 370/360	DCRT's Bldg 12 computer

At least four instruments can be operated simultaneously without need of an attendant in the main computer room. The system is now connected to all the instruments that it can service; hence new ones can be added only if present ones are disconnected.

A group of generalized data-processing programs has been developed to permit scientific users who are not computer experts to employ the system to:

1. Maintain their own files of experimental data on magnetic tape.
2. Perform simple mathematical operations on files. These include addition, multiplication, interpolation, least squares analysis into components and convolution.
3. Plot graphs of transformed data with india ink on tracing paper, thus producing line drawings suitable for publication without the need for the services of a medical illustrator.

The Building 2 computer now has in stock some 200 programs and subroutines written by local users. In addition to the four programmers: Marvin Shapiro, Jay Vinton, Luther Barden and Marie Chang, who have written some of the programs, four NIAMDD scientists are now actively contributing to the program library.

Significance to Biomedical Research and the Program of the Institute:

High speed digital computation of experimental data is vital to the conduct of much of the research conducted in LCP and LMB. This facility provides such needed on-line computer service and serves as a model for similar developments at NIH and elsewhere.

Proposed Course of Project:

A telephone data link will soon permit the system to act as a remote terminal for entering and receiving computer jobs from other computer centers at NIH and elsewhere. Continued development of the programs needed for a variety of applications is planned.

Publications:

Hirasawa, R., and Kon, H.: The electron paramagnetic resonance and polarized absorption spectra of Cu(II) doped single crystal of L-Histidine Hydrochloride Monohydrate. J. Chem. Phys. 56: 4467-4474, 1972.

Sharpless, N. E., Greenblatt, C. L., and Jennings, W. H.: Therapeutic interference studies: Mechanisms of interpolymer competition for dyes. Trans. N. Y. Acad. 35: 187-195, 1973.

Hagins, W. A., and Rüppel, H.: Fast photoelectric effects and the properties of vertebrate photoreceptors as electric cables. Fed. Proc. 30: 64-68, 1971.

Penn, R. D., and Hagins, W. A.: Kinetics of the photocurrent of retinal rods. Biophys. J. 12: 1073-1094, 1972.

Hagins, F. M.: Purification and partial characterization of the protein component of squid rhodopsin. J. Biol. Chem. 247: (in press).

Rüppel, H., and Hagins, W. A.: Spatial origin of the fast photovoltage in retinal rods. Symp. Biochem. Visual Pigm. (in press).

Yoshikami, S., and Hagins, W. A.: Control of the dark current in vertebrate rods and cones. Symp. Biochem. Visual Pigm. (in press).

Serial No. NIAMDD-LCP-4
1. Chemical Physics
2. Membrane Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Spectral, physical-chemical and photochemical properties of biologically active substances

Previous Serial Number: NIAMD-LPB-16

Principal Investigator: Norman E. Sharpless

Other Investigators: None

Cooperating Units: Frederick S. Brackett, Consultant, LCP-NIAMDD
Ralph G. Adams (LCP-NIAMDD)
William H. Jennings (LCP-NIAMDD)
Robert B. Bradley (LCP-NIAMDD)
James A. Ferretti (PSL-DCRT

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To investigate such molecular factors as structure, quantum parameters or physical properties in relation to the biological or pharmacological activities of the molecules.

Methods Employed:

These investigations utilize various forms of spectrophotometry (visible, UV, IR, NMR, EPR and fluorescence) which distinguish energy levels, and ORD, CD, gas chromatography and dielectric properties which distinguish other molecular properties, as well as theoretical quantum chemical calculations. Extensive use of computers is required to reach these objectives.

Major Findings:

Photochemistry of the Vitamin D System (with Dr. Brackett)

The program permitting the further solutions and plotting routines on the new computer has resulted in still further insights into the true mechanism of the photochemical sequence and products.

Chemistry and Physics of the Visual Process (with Dr. Adams and Mr. Jennings)

It was reported last year that retinaldehyde Schiff bases occur normally as a hydrate containing one molecule of water. Infra-red studies of retinaldehyde itself showed that there was no interaction between the carbonyl group and water to form a gem diol as in chloral hydrate or mesoxalic acid.

The Schiff base of retinaldehyde-ethanolamine phosphoglyceric acid is optically active. ORD studies show a negative Cotton effect curve, with inflexions at 450 nm (complex I) as well as in the 300-350 nm region. The sign of the rotation is opposite to and the extent of rotation is greater than that of the parent phospholipid.

Studies with retinaldehyde and di or tripeptides containing lysine and phenyl alanine are given in Dr. Adams' report.

Two papers from this project have recently been submitted for publication.

Nuclear Magnetic Resonance of Fused Ring Heterocyclic Compounds (with Dr. Ferretti and Mr. Bradley)

This work is completed and a paper concerning it has been submitted to Organic Magnetic Resonance.

The changes in spectra due to structural and electronic influences have permitted the determination of the enthalpy of dimerization of xanthydrol (5.8 kcals/mole) and the contribution of planarity to the ortho coupling constants nearest the heteroatoms (0.7 Hz).

Therapeutic Interference

Evaluation of fluorescence quenching constants of trypaflavine bound to DNA as well as other biopolymers show the trypaflavine to be bound to the gc pairs, and the Crystal Violet, bound as a dimer, quenches the fluorescence by a coupled oscillator process.

The synthesis of the spin labeled calcium binding complex (4-(2-(3-pentanedione-2,4)-2,2,6,6 tetramethyl piperidinooxyl) is unsatisfactory using the sodio derivative of acetylacetone, due to its sensitivity to bases. However, the corresponding thallium derivative shows great promise.

Significance to Biomedical Research and the Program of the Institute:

A proper understanding of the behavior of biologically active substances at the molecular level is absolutely necessary for the understanding and interpreting of their action at the cellular and organ level in order to evaluate their contributions to both healthy and diseased states.

Proposed Course of Project:

The study of the interactions of retinaldehyde with the model peptides will be continued, both in regard to structure and optical properties.

The spin-labeling of biologically important molecules will be continued.

Publications:

Sharpless, N. E., Greenblatt, C. L., and Jennings, W. H.: Therapeutic interference studies. Mechanisms of interpolymer competition for dyes. Trans. N. Y. Acad. Sci. 35: 187-195, 1973.

Sharpless, N. E., Adams, R. G., and Jennings, W. H.: Retinal Schiff bases. The effect of water on spectral absorption. Trans. N. Y. Acad. Sci. (in press).

Serial No. NIAMDD-LCP-5
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title:

1. π-π complexes of retinal with substances occurring in mammalian retina *in vivo*.
2. Construction of a model system to approximate the complex described and to determine a possible relationship to rhodopsin.

Previous Serial Number: NIAMD-LPB-7

Principal Investigator: Ralph G. Adams

Other Investigators: None

Cooperating Units: W. H. Jennings (NIAMDD-LCP)
N. E. Sharpless (NIAMDD-LCP)

Man Years:

Total:	1
Professional:	1
Others:	0

Project Description:

Objectives:

1. To determine the composition of the rhodopsin chromophore. Although we still consider our previously described complex II to be a prime candidate, recent reports of the absence of ethanolamines phosphoglyceride (EPG) in rhodopsin make essential the refutation of the evidence or finding an alternative candidate. To that end we are investigating lysine-containing peptides since in forming the required Schiff base only lysine and ethanolamine are considered.
2. The end point of a synthetic rhodopsin is still a valid objective and the route to that end is logically via the peptides, since rhodopsin is a lipo-protein.

Major Findings:

1. The evidence against the involvement of EPG in rhodopsin *in vivo* is two-fold: (1) that the spectrum of rhodopsin should change when

EPG's phosphate linkage to the glycerol backbone is cleaved by enzymatic action—it does not. However, I can reproduce the complex II spectrum by combining the cleavage products so that if complex II is the chromophore in vivo, the spectral criterion is false. (2) After the removal of 95.5% of EPG from rhodopsin, the extracted retinal is attached to lysine and since no spectral change occurred there could have been no trans-iminization from EPG to lysine. We can show theoretically that it makes no difference to complex II whether lysine or ethanolamine forms the Schiff base. Further, the di- and tri-peptides I have made, with lysine, form retinal Schiff bases whose spectra are indistinguishable from those formed by EPG.

The above work is currently being assembled in manuscript.

2. Previous attempts to work with bovine serum albumin (BSA) and retinal produced changes at 500 nm but the system is much too complicated. We feel that peptides containing alanine, lysine and phenylalanine produce more stable and clearer solutions. The N-t-BOC-l-lysyl-l-alanyl-l-phenylalanine ethyl ester, which appears to be helical, seems to be the most promising candidate. We have thus far only been able to produce the protonated Schiff base but feel that a complex II lysine may be possible.

Significance to Biomedical Research and the Program of the Institute:

To elucidate the structure of rhodopsin, the visual pigment is an obviously important step in understanding vision.

Proposed Course of the Project:

1. Application of results of kinetic studies to formation of model compounds approximating rhodopsin.
2. Formation of additional peptides to complex with retinal.
3. Rotatory dispersion and circular dichroism studies of synthetic peptide-retinal complexes.
4. Repeat other experiments with enzymes on rhodopsin.

Honors and Awards:

Invitation to address symposium on retina at NIH, June 1973.

Publications:

Sharpless, N. E., Adams, R. G. and Jennings, W. H.: Retinal Schiff bases. The effect of water on spectral absorption. Trans. N. Y. Acad. Sci. In press.

Serial No. NIAMDD-LCP-6
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Molecular structure determined by spectroscopic methods

Previous Serial Number: NIAMD-LPB-8

Principal Investigator: Edwin D. Becker

Other Investigators: Robert B. Bradley
Raj K. Gupta
Edwin E. Tucker

Cooperating Units: T. Phillip Pitner (NIH Fellow)
Roderick E. Wasylishen (NRCC Fellow)
H. Michael McIntyre (NBS)
W. Friauf, T. Clem (DRS-BEIB)
F. J. Brinley (Johns Hopkins University)
H. J. C. Yeh (NIAMDD-LC)
E. Gross (NICHHD)
J. S. Cohen (DCRT)
J. A. Ferretti (DCRT)
H. T. Miles (NIAMDD-LMB)

Man Years

Total: 5
Professional: 4
Other: 1

Project Description:

Objectives:

1. Development of an understanding of the forces within and between molecules, especially those of potential biological importance.

2. Development of spectroscopic methods for studying molecular structure and analyzing materials of chemical and biological interest.

Methods Employed:

The primary techniques used in this work are infrared spectroscopy (IR), Raman spectroscopy, and nuclear magnetic resonance spectroscopy (NMR). The three methods supplement each other in providing detailed information about the structure of molecules. In addition, the spectra are highly sensitive to the effects of molecular interactions. For studies of hydrogen bonding high precision vapor pressure measures provide information complementary to that obtained by the spectroscopic techniques. Digital computer methods are used extensively.

Major Findings:

A comprehensive infrared, NMR and vapor pressure investigation of hydrogen bonding in t-butanol has been completed. All of these data can be interpreted quantitatively in terms of an open (chain) trimer and a series of cyclic higher polymers. No appreciable dimer concentration was found, and probable reasons were advanced to account for the erroneous report of dimers in many previous studies. (Tucker, Becker)

Detailed interpretation of previously obtained infrared and Raman data for 1-methyluracil and a number of isotopic derivatives is underway. An "amide-II" band has been identified, contrary to previous reports in the literature that cis-amides display no amide-II band in the infrared. New IR data on oriented crystals and on mulls in the far IR have aided greatly in our efforts to complete detailed vibrational assignments. (Becker, Miles)

Apparatus has been developed for study of relatively rapid reactions (lifetimes of a few seconds) by Fourier transform (FT) NMR methods. Preliminary studies of the reaction of CNBr with N-acetylmethionine have been carried out. (Pitner, Becker) Further development of FT apparatus for the study of ^{13}C NMR has been carried out, and a new commercial FT accessory for the study of proton NMR at high magnetic fields has been put into operation. (Bradley, Pitner)

Interaction of cytochrome c, an electron transfer hemoprotein, with cytochrome c peroxidase (M.W. 36,000) was studied using the NMR technique. The studies provided direct evidence for a reversible 1:1 complex formation between cytochrome c and the peroxidase and indicated lack of heme-heme interaction in the complex. (Gupta)

Molecular orbital (INDO) calculations of nuclear spin-spin coupling constants in ammonia have been conducted as a function of HNH angle. Preliminary experimental measurements on several ^{15}N-anilines indicate that the observed coupling constants can be used with the calculations to estimate the planarity of the NH_2 fragment. (Wasylishen)

Significance to Biomedical Research and the Program of the Institute:

In this project we are attempting to obtain basic information on the structure and molecular interactions of nucleosides and their derivatives and to learn more about the nature of hydrogen bonding in these and other molecules. Such information may aid in an understanding of the relation between structure and function of nucleic acids and enzymes. In addition, the development of spectroscopic techniques applicable to molecules of biological activity and interest, such as steroids, alkaloids, pigments, etc., aids in the structure elucidation of many natural and synthetic substances studied throughout NIH.

Proposed Course of Project:

The vibrational assignment of 1-methyluracil will soon be completed, and normal coordinate calculations are planned.

A study of the equilibria involved in hydrogen bonding of a highly hindered alcohol, 2,4-dimethyl-3-ethyl-3-pentanol is in progress and should be completed soon. A comparison of these results with the previously obtained data for the unhindered alcohol t-butanol is expected to provide important information on hydrogen bonding.

A simple, but comprehensive, computer-aided analysis of the factors affecting pulse-Fourier transform NMR spectra has been started. This analysis is expected to simplify the choice of optimum operating parameters in FT-NMR spectroscopy.

Another NMR method closely related to the pulse-FT technique, correlation spectroscopy, is being investigated for use in studying aqueous solutions of biopolymers.

Publications:

Becker, E. D. and Farrar, T. C.: Fourier transform spectroscopy. Science 178: 361-368, 1972.

Miles, H. T., Lewis, T. P., Becker, E. D. and Frazier, J.: Identification of the amide II band in the cis-amide 1-methyluracil, in poly(U), and in poly(A)·poly(U). J. Biol. Chem. 248: 1115-1117, 1973.

Jennings, W. B., Boyd, D. R., Watson, C. G., Becker, E. D., Bradley, R. B. and Jerina, D. M.: The stereochemical dependence of ^{15}NCH and ^{13}CH coupling constants in oxaziridines. J. Am. Chem. Soc. 94: 8501-8504, 1972.

Gupta, R. K. and Yonetani, T.: NMR study of the interaction of cytochrome c with cytochrome c peroxidase. Biochim. Biophys. Acta 292: 502-508, 1973.

Gupta, R. K.: Electron transfer in cytochrome c. Role of the polypeptide chain. Biochim. Biophys. Acta 292: 291-295, 1973.

Yeh, H. J. C., Brinley, F. J., Jr. and Becker, E. D.: NMR studies on intracellular sodium in human erythrocytes and frog muscle. Biophys. J. 13: 56-71, 1973.

Tucker, E. E. and Becker, E. D.: Alcohol association studies. Part II. Vapor pressure, 220 MHz proton magnetic resonance and infrared investigations of t-butanol association in hexadecane. J. Phys Chem. In press.

Becker, E. D.: Nuclei other than hydrogen: a review of some nuclear properties and a discussion of their relaxation mechanisms. In Axenrod, T. and Webb, G. (Eds.): NMR of Elements Other Than Hydrogen. New York, Wiley-Interscience. In press.

Becker, E. D.: Introduction to pulse and Fourier transform methods. In Axenrod, T. and Webb, G. (Eds.): NMR of Elements Other Than Hydrogen. New York, Wiley-Interscience. In press.

REPORTED IN PRESS - 1972

Shoup, R. R., Becker, E. D. and Farrar, T. C.: The driven equilibrium Fourier transform NMR technique. An experimental study. J. Magnetic Res. 8: 298-310, 1972.

Becker, E. D., Shoup, R. R. and Farrar, T. C.: ^{13}C NMR spectroscopy: Relaxation times of ^{13}C and methods for sensitivity enhancement. Pure Appl. Chem. 32: 51-66, 1972.

Becker, E. D.: Some recent developments in high resolution NMR. Appl. Spectrosc. 26: 421-430, 1972.

Shoup, R. R. and Farrar, T. C.: ^{13}C magnetic relaxation rate studies of chloroform. J. Magnetic Res. 7: 48-54, 1972.

Serial No. NIAMDD-LCP-7
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Molecular Dynamics

Previous Serial Number: NIAMD-LPB-13

Principal Investigator: Ira W. Levin

Other Investigators: Robert A. R. Pearce

Cooperating Units: William C. Harris, Furman University, Greenville, South Carolina
O. W. Adams, National Science Foundation
S. Abramowitz, National Bureau of Standards

Man Years:

Total: 2
Professional: 2
Other: 0

Project Description:

Objectives:

An understanding of the vibrational characteristics of polyatomic systems through the analysis of Raman and infrared spectra, the determination of reliable intramolecular force fields and the interpretation of molecular charge distribution (intensity) parameters.

Methods Employed:

Various laser Raman and infrared spectroscopic procedures provided the required data for the vibrational analyses. Both the intramolecular force field determinations and large molecular orbital calculations were necessarily performed on the digital computer.

Major Findings:

1. Raman spectra of polycrystalline azomethane ($CH_3N=NCH_3$) and azomethane-d_6 were examined at liquid nitrogen temperatures using argon laser excitation. These data, in conjunction with a normal coordinate analysis, provided a basis for a complete vibrational assignment for the azomethane system. A 17 parameter molecular force

field, based upon the solid state vibrational frequencies of azomethane and its deuterated analog, was determined by least-squares procedures. The unusual coupling characteristics between the X-N stretching and X-N=N bending coordinates were examined in detail for both azomethane and a model system, trans-1,2-difluorodiazine (FN=NF). (Levin, Pearce and Harris)

2. Separate six parameter internal valence force fields were determined for cis- and trans-1,2-difluorodiimide (F-N=N-F), using a least-squares procedure. For the cis- and trans-1,2-difluoroethylenes (F-CH=CH-F), a seventeen parameter overlay internal valence force field was fitted to the in-plane vibrational frequencies, and separate four parameter general valence force fields were calculated for the out-of-plane vibrations. For both sets of molecules, potential energy distributions were examined with particular reference to the unusual couplings between the F-X stretching and F-X=X bending coordinates. These couplings were investigated in detail, especially for the 1,2-difluorodiimides, and it is concluded that they are predominately due to kinetic effects. (Levin and Pearce)
3. The vibrational spectra of trans-2-butene-d_0 and trans-2-butene-d_8 was recorded in the region from 4000 - 100 cm^{-1}. In conjunction with a normal coordinate analysis, a complete vibrational assignment was proposed on the basis of Raman depolarization values for the liquid samples, isotopic shift ratios and group coordinate considerations. Both assignments and vibrational coupling effects were examined in terms of the calculated potential energy distributions. A 21 parameter internal valence force field was determined by least-squares procedures from the observed polycrystalline frequencies of the two isotopic species. (Levin, Pearce and Harris)
4. The vibrational assignments and potential energy distributions for cis-2-butene were examined by transferring to the cis species a potential function from trans-2-butene and a model system, trans-1,2-difluoroethylene. The model system provides the crucial interaction force constants which account for effects occurring across the double bond and the skeletal out-of-plane force constants. The zeroth order frequencies for cis-2-butene calculated from the transferred potential function agree quite well with the low temperature Raman spectrum of the polycrystalline material. (Levin and Pearce)
5. A computer program was developed to refine a structure of a molecule given a suitable, simplified potential function and the totally symmetric a_g vibrational modes. Attempts were made to avoid using potential functions containing the less well-defined non-bonded atomic interactions. Moderate success was attained with several strained bicyclic systems, namely, 1,1,1-bicyclopentane, norbornane and norbornadiene. (Levin and Pearce)

Significance to Biomedical Research and the Program of the Institute:

Since experimentally determined vibrational frequencies and charge distribution parameters reflect the sum of several subtle electronic and nuclear contributions, force field and molecular orbital calculations permit both quantitative and qualitative descriptions of the dominant terms and create a link between empirical information and bond properties.

Proposed Course of Project:

Particular emphasis will be directed toward calculating molecular structures for moderately complex systems from both potential function information and spectral data. Specific questions will concern the limits of transferability of the various potential function models that are used in the structural refinements.

Publications:

Levin, I. W. and Harris, W. C.: Raman spectra of matrix isolated molecules: Application to the force field for CCl_4. J. Chem. Phys. 57: 2715-2721, 1972.

Müller, A., Mohan, N., Schmidt, K. H. and Levin, I. W.: A note on the comparison of the high low frequency separation with other approximation methods for the calculation of force constants. Chem. Phys. Lett. 15: 127-129, 1972.

Harris, W. C. and Levin, I. W.: Low temperature Raman spectroscopy of molecular solids. In Mathieu, J. P. (Ed.): Advances in Laser Raman Spectroscopy, Vol. I. London, Heyden and Son, Ltd. In Press.

Levin, I. W. and Harris, W. C.: Interpretation of the vibrational spectra of bicyclic hydrocarbons: norbornane and norbornadiene. Spectrochim. Acta. In press.

Pearce, R. A. R., Levin, I. W. and Harris, W. C.: Raman spectra and vibrational potential function of polycrystalline azomethane and azomethane-d_6. J. Chem. Phys. In press.

REPORTED IN PRESS - 1972

Harris, W. C. and Levin, I. W.: Raman spectra of polycrystalline acetone and acetone-d_6. J. Mol. Spectrosc. 43: 117-127, 1972.

Serial No. NIAMDD-LCP-8
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Chemistry and biosynthesis of natural compounds, and instrumental methods used in their study.

Previous Serial Number: NIAMD-LPB-17

Principal Investigator: Ulrich Weiss

Other Investigators: Kenner C. Rice

Cooperating Units: Dr. J. M. Edwards, School of Pharmacy, University of Connecticut, Storrs
Dr. J. V. Silverton, NHLI-LC

Man Years

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

Isolation in pure form, and elucidation of the chemical constitution of a variety of naturally occurring substances of chemical, biosynthetic, or biological interest. Study of instrumental techniques used in such work. Corroboration of chemical structure by total synthesis.

Methods Employed:

Standard procedures of organic chemistry, and instrumental methods (UV, IR, NMR spectroscopy). For measurement of optical rotatory dispersion and circular dichroism, the Cary 60 spectropolarimeter and its CD attachment are used.

Major Findings:

In continuing cooperation with Dr. J. M. Edwards and his students, three new constituents from the flowers of *Lachnanthes tinctoria* have been obtained; their chemical structure is under study. The unusual biosynthesis of the pigments of this plant is being studied with the help of ^{13}C magnetic resonance.

At the request of Dr. W. A. Hagins, work on the synthesis of a bis-nitroxyl radical designed to serve as a specific reagent for assay of Ca^{++} by ESR in biological systems has been undertaken. It had been planned to use as precursor of the nitroxyl radical a compound which had been prepared in 1897 from phorone and hydroxylamine. This product, "triacetonehydroxylamine," had always been believed to be N-hydroxy-2,2,6,6-tetramethyl-4-piperidone. However, the NMR spectrum of the substance is quite incompatible with this symmetrical structure, and on detailed examination, the compound has turned out to be hexahydro-3,3,7,7-tetramethyl-1,2-oxazepin-5-one. Very few representatives of this ring system had been prepared previously by rather involved methods. Data from 1897 indicate that the compound from phorone and hydroxylamine has strong pharmacological action (at least on the frog); the fact that our identification of its structure suggests convenient ways to prepare other compounds of this type may therefore be of interest.

The NMR spectrum of the compound shows that it consists at room temperature of one particular conformer; heating to 150° converts this complex spectrum to the simpler one expected for a completely flexible molecule. This aspect of the problem is being studied further by Dr. Rodney Wasylishen.

A compound $C_{13}H_{14}O_3$, obtained as a minor by-product of the reaction of dimethyl acetone dicarboxylate and glyoxal (see U. Weiss and J. M. Edwards, Tetrahedron Letters 1968, 4885) has been shown through x-ray crystallography by Drs. T. Akiyama and J. V. Silverton to be endo-tetracyclo[$5.5.1.0^{2,6}.0^{10,13}$]tridecane-4,8,12-trione. This structure had been anticipated from interpretation of the reactions which give rise to the compound. Only one representative of this very unusual system of four five-membered rings has been described prior to our work, and it is remarkable that our reaction should yield this system in one step from available, inexpensive starting materials. The compound has interesting potentialities for further elaboration to yield complex, conformationally rigid ring systems.

Biological tests of the quinone methides for antitumor action (see report for 1971-72) have not given positive results so far; the thiolcarbamates (ibid.) proved inactive in the antimalarial tests used at present.

Significance to Biomedical Research and the Program of the Institute:

Lachnanthes is said to have been used medicinally in earlier times, and its long-suspected photodynamic action has finally been established in the course of our work. The biosynthesis of its constituents, not yet fully understood and rather controversial, seems to depart from that of most other plant materials; it appears, however, that it typifies the biogenesis of a number of widely distributed natural substances. Use of ^{13}C resonance for elucidation of this biosynthesis would be an interesting new extension of this new technique.

The pharmacological action of our hexahydro-3,3,7,7-tetramethyl-1,2-oxazepin-5-one is being investigated at NHLI, and elsewhere; the transient, completely reversible paralysis of the extremities which has been observed in the frog suggests that the compound may have interesting pharmacological activities. Study of its conformation may be valuable for better understanding of the relation between molecular geometry and biological actions. There is some indication that rigidity of molecular structure may be helpful for pharmacological activity; the extremely simple synthesis of a representative of a new complex, rigid ring system may therefore lead to new, biologically active substances.

Proposed Course of Project:

The work on the pigments of Lachnanthes and their biosynthesis is to be continued. The synthesis of the nitroxyl reagent for Ca^{++} in biological systems is to be resumed, using other sources of nitroxyl. Research on the chiroptical properties of non-conjugated dienes will be continued, with particular attention to possibilities of collecting needed reliable information on the precise molecular geometry of such compounds by x-ray crystallography.

Publications:

Rice, K. C. and Weiss, U.: Identification of the so-called triacetonehydroxylamine as hexahydro-3,3,7,7-tetramethyl-1,2-oxazepin-5-one. Tetrahedron Letters. In press.

REPORTED IN PRESS - 1972

Edwards, J. M. and Weiss, U.: Quinone methides derived from 5-oxa- and 5-aza-9-phenyl-1-phenalenone in the flowers of Lachnanthes tinctoria (Haemodoraceae). Tetrahedron Letters: 1631-1634, 1972.

Edwards, J. M., Schmitt, R. C. and Weiss. U.: Biosynthesis of a 9-phenylperinaphthenone by Lachnanthes tinctoria (Haemodoraceae). Phytochemistry 11: 1717-1720, 1972.

Charney, E., Edwards, J. M., Weiss, U. and Ziffer, H.: Inverse sign of the chiral-optical effects of non-planar heteroannular cisoid dienes. Tetrahedron 28: 973-979, 1972.

Serial No. NIAMDD-LCP-9
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Titles:

1. A study of photoreactions and the mechanisms by which these reactions occur.
2. An investigation of the absolute stereochemistry of several dihydro diols obtained from microbial oxidation of aromatic substrates.
3. A study of the interdependence of CD curves and the structure and stereochemistry of dienes.

Previous Serial Number: NIAMD-LPB-18

Principal Investigator: Herman Ziffer

Other Investigators: Jeffrey I. Seeman

Cooperating Units: Donald M. Jerina (NIAMDD-LC)
Herman J. C. Yeh (NIAMDD-LC)
D. T. Gibson, University of Texas

Man Years:

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

1. To examine the photochemistry of a number of β,γ-unsaturated ketones in order to learn more about the mechanism and stereochemistry of their reactions.
2. To determine the absolute stereochemistry of several dihydro diols, microbial metabolites of aromatic substrates, in order to learn more about the enzyme-substrate complex.
3. To prepare unstable Z isomers of N-alkyl aldimines in order to study the mechanism by which they isomerize to the more stable E isomer.

Methods Employed:

The Cary 60 spectropolarimeter has been used to obtain ORD and CD measurements. Standard organic chemical techniques were employed for the synthesis and proof of structure of a number of compounds. Along with the usual spectroscopic measurements (NMR, IR and UV) we have used the Varian time averaging computer in conjunction with our NMR equipment to obtain several important NMR measurements necessary for structure determinations.

Major Findings:

1. The absolute stereochemistry of a microbial metabolite of toluene has been determined as (+) cis-1(S), 2(R)-dihydroxy-3-methyl cyclohexa-3,5-diene by chemical and physical methods. The agreement between the chemical and physical methods will now permit us to employ the latter (c.d. of the reduced diol dibenzoate) to determine the structure of several metabolites (dihydro diols) that can not be converted to compounds of known absolute stereochemistry.
2. The stereochemistry previously assigned by other workers to the major and minor photoproducts of A-homo-cholestan-3-one has been shown to be reversed. The structures of these photoproducts were established by an unequivocal synthesis. The stereochemistry of these products is critical in understanding both the singlet and triplet photochemistry of the β,γ-unsaturated ketones.
3. The major (5β-vinyl-A-nor-cholestan-3-one) and minor (5α-vinyl-A-nor-cholestan-3-one) photoproducts described above were shown to undergo oxa-di-π-methane rearrangement upon sensitized (acetone) irradiation. The rearrangement was stereospecific and proceeded with retention of stereochemistry at the carbon between the carbonyl and double bond.
4. A photochemical method for enriching solutions of N-alkyl aldimines in the thermodynamically less stable Z isomers has been developed. The E and Z isomers of the aldimine from ^{13}C-9-anthraldhyde and ^{15}N-t-butylamine were prepared and the reduced coupling constants $^{1}K(H-^{13}C)$, $^{1}K(^{13}C=^{15}N)$ and $^{2}K(H-C=^{15}N)$ determined. The cis geometry between the hydrogen and the lone pair of electrons in nitrogen (Z isomer) results in a large positive contribution to K; a result consistent with "through space" coupling.

Significance to Biomedical Research and the Program of the Institute:

Information on the absolute stereochemistry of metabolites will assist in developing models which describe the way substrates attach to enzymes and the course of the reaction.

The increased knowledge derived from studies of the mode and mechanism of photochemical reactions permit the synthesis of compounds that are difficult to prepare by other means.

Proposed Course of Projects:

1. To determine and compare the absolute stereochemistry of a number of cis dihydro diols produced by microbiol metabolites of aromatic substrates.
2. To examine the photochemistry of $\Delta^{9,10}$1-methyl octal-2-one in an effort to distinguish between a concerted and stepwise mechanism for the oxa-di-π-methane rearrangement.
3. To examine the photochemistry of several substituted $\Delta^{9,10}$-octal-2-ones in order to determine the kinetic and geometric features which determine the photochemical reaction path of β,γ-unsaturated ketones.
4. To examine the effects of substituents on the rates of isomerization of N-alkyl aldimines in order to study the mechanism of the isomerization about the carbon to nitrogen double bond.

Publications:

Yeh, H. J. C., Ziffer, H., Jerina, D. M. and Boyd, D.: Stereochemical dependence of the sign and magnitude of coupling constants in geometry in ^{15}N-E and Z aldimines. J. Am. Chem. Soc. 95: 2741-2743, 1973.

Ziffer, H., Jerina, D. M., Gibson, D. T., and Kobal, V. M.: Absolute stereochemistry of the (+)-cis-1,2-dihydroxy-3-methylcyclohexa-3,5-diene produced from toluene by *Pseudomonas putida*. J. Am. Chem. Soc. In press.

Serial No. NIAMDD-LCP-10
1. Chemical Physics
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effects of altitude (hypoxia), exercise, and other environmental stresses on physiological, biochemical and pathological mechanisms in animals.

Previous Serial Number: NIAMD-LPB-19

Principal Investigator: Paul D. Altland

Other Investigators: Mr. H. F. Brubach, Mr. M. G. Parker, and Dr. R. G. Sellner

Cooperating Units: Dr. Benjamin Highman, Section on Pathologic Anatomy, Laboratory of Experimental Pathology, NIAMDD, LEP-2 A.

Man Years

Total:	6
Professional:	4
Other:	2

Project Description:

Objectives:

To determine physiological, biochemical, and pathological changes occurring during exposure to hypoxia induced by decompression or by low oxygen concentrations in nitrogen. To determine the mechanism of action of cross-adaptation between altitude-acclimation and exercise and between cold-acclimation and hypoxia. To determine the effects of exercise and exogenous pituitary and adrenal hormones on the susceptibility of rats to experimental bacterial endocarditis (Dr. Altland). To determine the mechanism involved in the production of deleterious effects of decompression hypoxia upon gestation (Dr. Sellner).

Methods Employed:

Biochemical analysis of serum enzymes and metabolic end products. Physiological measurement of respiration, body temperature, exercise performance and hypoxic tolerance. Histopathological methods for demonstration of fatty and necrotic changes in vital organs. Quantitation of serum levels of circulating steroid hormones by radioimmunoassay. Operation of two altitude chambers and two animal exercisers.

Major Findings:

In experiments on the effects of cross-adaptation between cold and altitude (hypoxia) it was found that cold-acclimated rats were relatively more intolerant to hypoxia (6.6 or 4.9% O_2 in N_2) at an ambient temperature of 1.7°C than unacclimated rats. Cold-acclimated rats showed relatively greater increases in serum concentrations of glutamic oxalacetic transaminese (SGOT), glutamic pyruvic transaminase (SGPT), aldolase (SALD) and lactic dehydrogenase (SLDH) immediately after exposure to 4.9% O_2 and a greater concentration of SGPT at 6.6% O_2 than unacclimated rats. Serum urea nitrogen, plasma corticosterone, serum glucose, and blood lactate were similarly elevated in both groups at both hypoxic levels. The cold-acclimated rats had a higher incidence of renal tubular dilatation, fatty changes in striated muscles, and severe hepatic glycogen depletion. All rats showed myocardial inflammatory foci one day after exposure which persisted for 4 days. Cold-acclimated rats maintained higher body temperatures during exposure. The reduced tolerance of the cold-acclimated rats is attributed to an increased metabolism which increased tissue hypoxia.

In a study to determine the effect of exercise and exercise training on the susceptibility of epinephrine-treated rats to endocarditis it was found that exercise significantly increased the incidence of endocarditis and that training reduced the incidence and severity of the infection. The training apparently improved the cardiac microcirculation and permitted adequate tissue perfusion to improve resistance to the disease. (Altland)

In experiments on the effects of hypoxia on gestation it was found that rabbits exposed to 16,000 ft. simulated altitude (barometric pressure 412 mm Hg.; pO_2 84.8 mm Hg.) exhibited a greater incidence of fetal resorption, spontaneous premature termination of pregnancy, and decreased fetal size than unexposed controls. (Sellner)

Significance to Biomedical Research and the Program of the Institute:

It was postulated previously that homeostatic mechanisms which are adequate in singly stressed animals become inadequate or exhausted in those dually stressed. Unacclimated rats dually stressed (exposed to cold and hypoxia simultaneously) survived better and showed fewer physiological and pathological changes than cold-acclimated rats. This clearly shows that the above postulate does not apply to cold-acclimated rats exposed to cold and hypoxia and that there is actually a negative cross-acclimation between cold and hypoxia. This principle may be of importance to individuals residing temporarily or permanently at high altitude or in cold environments.

There are a number of well established factors which predispose animals and man to bacterial endocarditis such as rheumatic fever, congenital heart disease, coronary atherosclerosis, hyperimmunity, and post surgical trauma. In our laboratory we have found that environmental stresses such as altitude (hypoxia), cold and exercise are also important contributing factors. Furthermore we have shown that physical training has beneficial effects on reducing the incidence of endocarditis in epinephrine-treated rats, which suggests that good physical condition may aid in diminishing susceptibility to endocarditis in other animals or possibly in man.

Elucidation of the mechanism by which fecundity is reduced by hypoxia may contribute to a better understanding of the interrelationships of the fetus, placenta, and hypothalamic-hypophyseal tract in the maintenance of pregnancy.

Proposed Course of Project:

Study will be completed concerning the effects of severe cold exposure of rabbits on tolerance and on the mechanisms which produce alterations in tissues, serum enzymes, lactate, corticosterone and other clinical parameters. Experiments will be conducted on the effects of cross-adaptation between altitude and exercise at room temperature and in the cold. Investigations will be continued on the effects of exercise on the susceptibility of rats to experimental bacterial endocarditis with emphasis on the possible effects of adrenocorticotropin (ACTH) and adrenal cortical hormones (Dr. Altland). Studies will be continued on the mechanism responsible for altitude induced effects on reproduction with emphasis on serum levels of steroid hormones (Dr. Sellner).

Publications:

Dieter, M. P., Altland, P. D., and Parker, M. G.: Serum and tissue enzymes and adrenocortical responses in rats exercised at intervals after cold-acclimation. Can. J. Physiol. Pharmacol. 50: 717-721, 1972.

Highman, B., and Altland, P. D.: Effects of exercise and training on susceptibility of epinephrine-treated rats to bacterial endocarditis. Life Sci. 11: 931-939, 1972.

Altland, P. D., Highman, B., and Sellner, R. G. (with technical assistance of M. G. Parker and H. F. Brubach): Tolerance of cold-acclimated and unacclimated rats to hypoxia at 1.7°C. Int. J. Biometeor. 17: (in press).

REPORTED IN PRESS - 1972

Altland, P. D., Highman, B., and Dieter, M. P. (with technical assistance of M. G. Parker and H. F. Brubach): Reduced hypoxic tolerance of cold-acclimated rats: serum enzymes and tissue changes. Am. J. Physiol. 222: 1441-1445, 1972.

Serial No. NIAMDD-LCP-11
1. Chemical Physics
2. Spectroscopy & Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Molecular structure, organization and intermolecular forces

Previous Serial Number: NIAMD-LPB-9

Principal Investigator: Elliot Charney

Other Investigators: H. James Hofrichter

Cooperating Units: Dr. Kenneth Foster, Naval Medical Research Institute
Dr. Daniel Glaubiger, Stanford University Medical School
Dr. Kurt Kohn, NCI

Man Years:

Total:	4
Professional:	2
Other:	2

Project Description:

Objectives:

The general objectives of this research are to determine the role of molecular structure and of intermolecular forces in chemical, physical and biological phenomena by exploring the optical properties of molecules (spectroscopic absorption, optical rotatory dispersion, circular and linear electric-field induced dichroism).

Methods Employed:

In addition to the methods previously employed, we have now constructed and have in operation a continuous recording microspectropolarimeter capable of measuring the optical activities of samples as small or smaller than 5 microns in diameter. This device which incorporates the microspectrophotometer reported previously, also is capable of measuring the linear dichroism, linear birefringence and circular dichroism of these samples. It uses a piezo-electric modulator modulated at about 66 kilo-Hertz. While the system is capable of measuring the optical activity of small isotropic samples, e.g. a 5 micron diameter film of a solution, it was designed for the purpose of measuring the optical activity of oriented and partially oriented samples; in particular of the "pseudo-crystals of acid polyribosadenylic acid (poly A).

Major Findings:

1. Electric field induced properties of large molecules

    a. DNA: There is still much uncertainty about the physical and structural properties of DNA. Our earlier electro-optic measurements on calf-thymus DNA have opened the question of the orientation of the nucleoside bases in the DNA double helix in solution. New measurements of the temperature dependence of the electric dichroism and of the optical activity of double helical DNA (i.e. above the melting point) sonicated to a molecular weight of 125,000 demonstrate that the molecule in solution exhibits the characteristic of a rigid-rod whose orientation in an electric field is largely determined by the dipole moment induced by the electric field. The permanent moment component, if any, appears small. This very likely accounts for the orders of magnitude variation in the values of the dielectric properties which have appeared in the literature.

    b. Field effects on the stability of biological helical polymers: (with Dr. Foster)

    In our studies on DNA and on polybutylisocyanates, we proved that the large electric fields (up to 35 kilovolts/cm) used in those experiments did not change the structure of those molecules. Nevertheless, there have been persistent reports that helical polymers maintained at the edge of helix stability, for example at or near their melting points, are or would be affected by fields such as these. We are currently studying this possibility using poly-γ-benzyl L glutamate (PBLG). Thus far measurements of PBLG of 75,000 molecular weight do not substantiate these predictions. Since the effect is expected to increase at least linearly with molecular weight, we are currently examining PBLG with a molecular weight of 550,000.

    c. Binding of antibiotics to DNA (with Dr. Glaubiger and Dr. Kohn):

    The binding to DNA of the dyes anthramycin and actinomycin D both of which exhibit known antibiotic activity have been studied by electric dichroism. In the case of anthramycin, which binds to DNA with extraordinary strength (Kohn and Specus. J. Mol. Biol. 51, 551 (1970), the transition moment of the 340 mμ band is approximately 41° from the helix axis. The structural implications of this result are now being analyzed, the most likely implication being that anthramycin does not intercalate the base pairs of DNA unlike most of the other more or less planar antibiotics which do intercalate.

2. Other optical studies on large molecules

a. Polynucleotides (with Dr. Hofrichter):

The anomaly found in the electro-optical properties of both Poly A and DNA which has led to the question of the orientation of base pairs in the helices in solution should lead to distinct effects in the optical activity which may be most readily observed in oriented or partially oriented samples. We are planning to measure the optical activity of "pseudo-crystals" (Zimmerman, Steven B. and Coleman, Norman, F.: An ordered precipitate of polyadenylic acid formed by freezing at acidic pH: Comparison of X-ray diffraction and other properties of the precipitate with those of fibers or direct acid-precipitates. Biopolymers 2: 1943-1960, 1972.) of double helical acid poly A using the microspectropolarimeter described under methods.

b. DNA (with Dr. Hofrichter):

Another method of pursuing the same question described above with respect to poly A is to measure the linear dichroism of fibres of DNA which can also be studied by X-rays. All of our hard knowledge of the base orientation in DNA now comes from X-ray scattering. Preliminary measurements of the linear dichroism of DNA fibres have produced values which are more in accord with the solution measurements than with the Watson-Crick-Wilkins Structure. However, it has been difficult to produce known B-form fibres and this problem is still under attack.

Significance to Biomedical Research and the Program of the Institute:

Optical and electrical properties of small and large molecules helps elucidate their structures and the relationships between structure, reactivity, and the intermolecular forces.

Proposed Course of Project:

We will continue to study the structural question arising from the electro-optical investigation of DNA. In addition, we expect to study with Dr. Kiwamu Yamaoka of the University of Hiroshima, Japan, the relaxation properties of DNA in solution and as well, the general applicability of existing theories of relaxation processes to polyelectrolyte polymers. We hope also to extend our capability of measurement of both crystal and electric dichroism into the near vacuum ultraviolet region so as to be able to study the electronic transitions of the peptide and nucleoside chromophores in that region. In the case of the peptide the transitions in this region dominate the optical properties at longer wavelengths.

We also plan to investigate, if time and facilities permit, a number of biologically interesting phenomena associated with protein behavior in transcription, using the electric dichroism technique. Finally, we have under consideration a theoretical investigation of two aspects of the optical phenomena observed in the nucleic acid polymers, the effects of interactions of transitions in dinucleosides of fixed conformation on their magnetic circular dichroism and a theoretical calculation of the effect of the helically anisotropic phosphate-ribose backbone on the electronic transitions of the nucleoside bases in DNA and poly A.

Publications:

Charney, E., Edwards, J. M., Weiss, U. and Ziffer, H.: Inverse sign of the chiral-optical effects of non-planar heteroannular cisoid dienes. Tetrahedron 28: 973-979, 1972.

Yamaoka, K. and Charney, E.: Electric dichroism studies of macromolecules in solutions. I. Theoretical considerations of electric dichroism and electrochromism. J. Am. Chem. Soc. 94: 8963-8973, 1972.

Yamaoka, K. and Charney, E.: Electric dichroism studies of macromolecules in solutions. II. Measurements of linear dichroism and birefringence of DNA in orienting electric fields. Macromolecules 6: 66-76, 1973.

Charney, E.: Electro-optics in the infrared region. In O'Konski, C. T. (ed.): Molecular Electro-Optics. New York, New York, Marcel Dekker. In press.

Serial No. NIAMDD-LCP-12
1. Chemical Physics
2. Spectroscopy & Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Conformation and electronic structure of biological molecules

Previous Serial Number: NIAMD-LPB-10

Prinicpal Investigator: William A. Eaton

Other Investigators: Dr. David G. Hendricker

Cooperating Units: Dr. Philip J. Stephens (Univ. of Southern California)
Dr. Marvin W. Makinen, formerly NIDR-LB
Dr. Walter Lovenberg, NHLI-ET
Dr. H. James Hofrichter

Man Years

Total:	1.3
Professional:	1.3
Other:	0

Project Description:

Objectives:

The general aim of the research is to relate the stereochemistry and electronic structure of biological molecules, particularly proteins, to their biochemical reactivity and function.

Methods Employed:

In addition to conventional biochemical spectroscopic methods, techniques have been developed for obtaining polarized spectra of microcrystals and single cells from the ultraviolet to the near infrared wavelength regions.

Major Findings:

1. Heme proteins (with Drs. Stephens and Makinen)

   a. Electronic structure of heme. We have investigated the optical spectra of a variety of heme protein derivatives from measurements of linear dichroism of single crystals and natural and

and magnetic circular dichroism of solutions. From the single crystal results we have found the directions of the electric dipole transition moments, from the natural circular dichroism the relative magnitudes of the magnetic dipole transition moments, and from the magnetic circular dichroism the permanent magnetic moments of the excited states. With this extensive characterization of the excited states we can make convincing spectroscopic assignments.

We have found that the pattern of excited electronic states, as well as the differences in patterns for derivatives with different ligand, spin, and oxidation states of the iron, agrees quite well with predictions of extended Huckel calculations. With the oxygen oriented in the Griffith geometry we can correlate the observed and predicted frequencies and polarizations of charge-transfer type excitations into and out of (mainly) oxygen molecular orbitals. These transitions should be sensitive to heme-oxygen stereochemistry. Thus, a comparison of our experimental results with new calculations that include a variety of oxygen configurations should lead to the correct structure.

b. Conformation. We have completed a study of the conformation of the heme pocket of hemoglobin by measuring changes in porphyrin plane orientation using polarized single crystal spectroscopy. The results not only show that porphyrin-globin contacts change upon oxygenation, but they also provide important supporting evidence for the Hoard-Perutz hypothesis that the iron-porphyrin displacement is the stereochemical trigger for cooperative oxygen binding.

2. Sickle Cell Anemia (with Drs. Hofrichter and Hendricker)

We have begun an investigation of the structure of the microtubules that deform the erythrocytes in patients with sickle cell anemia. The spectroscopic information, as well as the experimental techniques developed in the single crystal studies, have provided the basis for investigating the orientation of the hemoglobin molecules within the microtubule. Our polarized absorption measurements on single sickled erythrocytes restrict the possible orientation of the long axis of the hemoglobin molecule to within 20° of the long axis of the microtubule. This result suggests that longitudinal contacts between molecules do not contain the mutation sites, and that the effect of the mutation is exerted in the lateral aggregation of the molecules within microtubules, or in the lateral aggregation of the microtubules to form crystalline-like domains.

3. Iron-sulfur proteins (with Dr. Lovenberg)

We have completed a theoretical and experimental study on the stereochemistry and electronic structure of the iron sulfur complex in rubredoxin. We find that our optical data (single crystal absorption, low temperature absorption, natural and magnetic circular dichroism) and the spectroscopic results of others (electron and nuclear magnetic resonance, Mossbauer, and Raman spectroscopies) can be rationalized in terms of a distorted tetrahedral complex in both oxidized and reduced rubredoxin. It is not possible, however, to correlate the distortion measured by the various spectroscopic methods with that found by X-ray diffraction studies at 1.5 Å resolution.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of relationships between conformation and electronic structure of the active sites of proteins should lead to a much deeper understanding of how these molecules perform their biological function.

A knowledge of the structure of the sickle cell microtubule should be important for designing methods of preventing the disease.

Proposed Course of Project:

We shall continue to investigate the electronic structure and conformation of heme proteins. With the new general phase modulation microspectrophotometer we will measure circular dichroism on single crystals. The oriented circular dichroism should enable us to make further spectroscopic assignments and thereby provide new insights into the origin of heme protein spectra. We will compare our experimental results with new theoretical calculations being carried out in Professor Martin Gouterman's laboratory at the University of Washington.

In collaboration with Dr. Eduardo Padlan (NIAMDD-LMB) we shall attempt to solve the structure of the heme-oxygen complex in oxyhemoglobin and oxycobaltmyoglobin by X-ray diffraction.

We plan to explore additional optical properties of sickle cells and gels, principally natural circular dichroism, in an attempt to elucidate the remainder of the structure of the microtubule.

Publications:

Cheng, J. C., Osborne, G. A., Stephens, P. J. and Eaton, W. A.: Infrared magnetic circular dichroism in the study of metalloproteins. Nature 241: 193, 1973.

Makinen, M. W. and Eaton, W. A.: Polarized single crystal absorption spectra of carboxy- and oxyhemoglobin. Ann. N. Y. Acad. Sci. In press.

Eaton, W. A. and Lovenberg, W.: The iron-sulfur complex in rubredoxin in Iron-Sulfur Proteins, edited by W. Lovenberg, Academic Press. In press.

Serial No. NIAMDD-LCP-13
1. Chemical Physics
2. Spectroscopy & Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Structure and interaction of paramagnetic biomolecules

Previous Serial Number: NIAMD-LPB-12

Principal Investigator: Hideo Kon

Other Investigators: None

Cooperating Units: Dr. Daniel W. Nebert, NICHHD-LBS
Dr. Albert Szent-Györgyi, Marine Biological Labs, Woods Hole, Massachusetts

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To investigate the electronic structure and the chemical behavior of paramagnetic molecules which occur in biological systems, or which serve as a suitable model for a natural system.

Methods Employed:

Electron paramagnetic resonance (epr) was the principal experimental means. However, any other methods, spectroscopic or otherwise, have been used whenever the objective calls for one. The experimental results were interpreted mainly on the basis of molecular quantum theory.

Major Findings:

1. (Continued from item 2 of 1972 report)

Theoretical treatment of the observed epr absorption of molecular oxygen in various gas matrices was made in order to assess the nature of the environment for oxygen. It was found that an oxygen molecule in such a matrix is carrying out the zero-point torsional oscillation.

The height of the potential barrier is 152 cm^{-1} in N_2 and 135 cm^{-1} in CO matrix. The extreme spectral broadening which hindered such observation in the past was found to originate from imcomplete averaging of fluctuations in the polar as well as the azimuthal angles in the motion. Analysis of the O^{17} hyperfine structure indicates that the valence shell electrons of oxygen are not perturbed in N_2 matrix from the gaseous state.

2. Study by epr of the nitric oxide complex of Fe(II) meso-tetraphenylporphine (TPP) in various solvents and conditions has been under way. The objective is to assess the effect of hydrophobicity in heme crevice on the epr absorption and thereby facilitate the interpretation of such spectra from the biological analogues under various conditions. As the first necessary step, the conditions for the synthesis of authentic pentacoordinated NO.TPP.Fe(II) was established by the combination of epr and mass spectrometric observations of pyrolysis of NO.TPP.Fe(II).pyridine.

3. With Dr. Nebert

The characteristic epr signal with a set of g value (8.0, 3.7, and 1.7) from cytochrome P-450 in some mammalian microsome preparations was studied at 9°K and simultaneously by the optical absorption at room temperature. This particular signal is due to a high-spin state of the Fe(III) hemoprotein, and is inducible _in vivo_ by aromatic hydrocarbons and phenobarbital injection, but it is not necessarily correlated with aryl hydrocarbon hydroxylase induction. It was established from this study that aromatic hydrocarbon treatment of _only genetically responsive strains_, rather than of any animals, causes an enhancement of P-450 with this particular spin state.

4. With Dr. Szent-Györgyi

The interaction of amines with ketone aldehydes or unsaturated aldehydes, which normally leads to the Schiff's base formation, has been known to exhibit a transient absorption in the visible region. Study of the same systems by epr also demonstrated the transient signal which can be qualitatively correlated with the optical absorption. It is, therefore, concluded that the interaction of amines with aldehyde causes an electron transfer between them in the ground state. Sulfur compounds, such as glutathione, was found to accelerate the transfer when added to the system in minute quantity.

Significance to Biomedical Research and the Program of the Institute:

These investigations contribute basic information toward understanding interactions involving metal ions, small organic molecules and the protein in more complicated and biologically significant systems.

Proposed Course of Project:

Investigations of the relationship between the function and the structure of metalloproteins will be continued along with the study of the structure of small paramagnetic molecules.

Publications:

Makinen, M. W., Milstien, J. B. and Kon, H.: Specificity of interaction of haptoglobin with mammalian hemoglobin. Biochem. 11: 3851-3860, 1972.

Kon, H.: Paramagnetic resonance of molecular oxygen in condensed phases. J. Am. Chem. Soc. 95: 1045-1049, 1973.

Nebert, D. W. and Kon, H.: Genetic regulation of aryl hydrocarbon hydroxylase induction. J. Biol. Chem. 248: 169-178, 1973.

Kon, H., Szent-Györgyi, A.: Interaction of amines with ketone aldehydes and unsaturated aldehydes. Proc. Nat. Acad. Sci. USA. In press.

Nebert, D. W., Considine, N. and Kon, H.: Genetic differences in cytochrome P-450 during induction of mono-oxygenase activities. Drug metabolism and disposition. In press.

REPORTED IN PRESS - 1972

Hirasawa, R. and Kon, H.: Electron paramagnetic resonance and polarized absorption spectra of Cu(II) doped single crystal of L-histidine hydrochloride monohydrate. J. Chem. Phys. 56: 4467-4474, 1972.

Serial No. NIAMDD-LCP-14
1. Chemical Physics
2. Spectroscopy & Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Electronic and molecular structural investigations

Previous Serial Number: NIAMD-LPB-14

Principal Investigator: Ruth McDiarmid

Other Investigators: None

Cooperating Units: None

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To describe the electronic and molecular structures of ground and excited states of simple molecules.

Methods Employed:

The measurement and analysis of electronic absorption spectra, predominantly in the ultraviolet. The calculation, with digital computers, of energy levels and geometries to correlate with and further explain the experimental observation.

Major Findings:

1. The ultraviolet spectra of WF_6 and MoF_6 were measured for correlation with the spectra of ReF_6, previously obtained. At energies lower than 1500 Å all three molecules show the same number and pattern of absorption bands, differing only in the energies of the bands. The assignment of all three bands in each molecule must be the same: Fluorine → unfilled E_g metal orbital. The anticipated d-d transition of ReF_6 is not observed.

2. The spectrum of $(CH_3)_2S$ was investigated from 3000-1720 Å in hydrocarbon and trifluorethanol solutions and from 3000-1520 Å in the vapor phase. At least 9 different absorption systems, each composed of many vibronic bands, were observed. Of these systems at least 6 belong to molecular Rydberg series and can be directly correlated with bands observed in the spectrum of H_2S. Two of the others most probably arise from transitions from the second highest filled orbital, one of the few reports of such a type of transition in a saturated molecule.

Significance to Biomedical Research and the Program of the Institute:

This research project seeks to understand the electronic changes a molecule undergoes on absorbing light and, more significantly, the structural alterations that a given electronic change can cause. This study can, if fruitful, provide a basis for a physical interpretation of the relative spectroscopic results obtained in other, more general studies.

Proposed Course of Project:

1. The spectra of the metal hexafluorides will be extended beyond 1500 Å in an attempt to find the transitions arising from promotions to the metal t_{2g} orbital so that the theoretically desired $e_g - t_{2g}$ energy separation can be estimated.

2. Now that a suitable instrument has been purchased and installed, the ∿1890 Å transition of diethyl ether will finally be reinvestigated.

3. The study of $(CH_3)_2S$ will be completed by a measurement and analysis of the spectrum of the deuterated compound to confirm the analysis of the light compound.

4. It is hoped that the remaining aspects of the ongoing ethylene investigation can be resolved.

Publications: None

Serial No. NIAMDD-LCP-15
1. Chemical Physics
2. Spectroscopy & Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mechanisms of energy transfer at cellular sites of photochemical action.

Previous Serial Number: NIAMD-LPB-15

Principal Investigator: Rodney A. Olson

Other Investigators: None

Cooperating Units: Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada.

Man Years*

Total	1
Professional:	1
Other:	0

*The principal investigator commenced a foreign assignment as Visiting Professor in the Department of Biology at Dalhousie University on July 1, 1972. Scientific equipment and apparatus for this project arrived at Dalhousie on August 4, 1972. Four days later on August 8, 1972, the principal investigator was stricken with bacterial endocarditis and was subsequently hospitalized until late November, 1972. A period of convalescence followed and actual physical work on the project began some time in early January.

Project Description:

Objectives:

To interpret the mechanisms of energy transfer and allied metabolic steps at cytological sites of photochemical action. To determine the role of chromophore orientation in the laminar photoreceptors (chloroplasts, etc.) and in crystalline pigment-protein systems and paracrystalline pigment-lipid systems simulating the organized structure of the photosynthetic apparatus.

Methods Employed:

Low temperature (77°K) microspectrometry is used to sharpen spectra and reveal components which are obscured by vibrational band-broadening at room temperature. The microcryostat-spectrometer system described in an earlier report has been reassembled at Dalhousie and a number of refinements in design have been incorporated.

The electronic on-line digital timing and data transforming circuitry described in the previous report has now been completed by the NIH Biomedical Engineering Branch and is currently being tested and evaluated here at Dalhousie on the above described cryostat-spectrometer system.

Major Findings:

Large crystals (0.5 x 1.5 mm) of bacteriochlorophyll-protein have been grown for measurements of low temperature polarization spectra. While these specimens offer an ample cross-sectional area for convenient measurements in the cryostat-microspectrophotometer with low power special optics of long working distance, the optical density in the 800 nm. region is so great that noise prevails over the signal. This difficulty was overcome by cutting thin sections (3-5 micrometers) of frozen crystals in the B&L "Cryocut" microtome. Low temperature polarization spectral measurements of these specimens and those in vivo chloroplast specimens described by manual observations in the previous report are currently being recorded and evaluated by the on-line data transforming circuitry.

Proposed Course of the Project:

The microcryostat-spectrophotometer system has been fitted with alternate high pressure compact-arc light sources for the measurement of fluorescent "glow curves" on warming and the low temperature fluorescence excitation spectra of in vivo chloroplasts. It is hoped that this work can be done here at Dalhousie with appropriate local species with large and ideal chloroplasts.

Publications: None

LABORATORY OF PHYSICAL BIOLOGY

Our migration to Building 6 has happened to coincide with new departures in most of the Laboratory's research units. This is, therefore, an appropriate occasion to summarize the backgrounds of the several investigative threads that will henceforth be the fabric of Physical Biology and indicate how they relate to each other and to the NIH mission.

From the first days of biomedical science it has been recognized that human birth in which the fetus leaves the dark aquatic womb to take up individual life involves many exquisitely programmed anatomical and physiological miracles -- awareness of the outside world via sight, hearing, and the other senses; conversion to air-breathing; the genesis of locomotion; transfer to independent feeding and digestion. Human development thus comprises aspects of nearly all the processes which engage biomedical research. Among the infinity of inquiries into these mysteries are those occupying LPB: the nature and integration of chemical changes during maturation, biological scheduling, processes involved in intake and exclusion of dissolved material by cells, the mechanism of muscular contraction, intracellular architecture, and the structure and ordering of certain key biological macromolecules.

For many years Dr. Levenbook's group has been investigating metamorphosis, which is an exaggerated model of human development because it results in the spontaneous transformation of one type of independent organism into another. One classic example is the conversion of the wriggling, gill-breathing, vegetarian tadpole into the hopping, air-breathing carnivorous frog. The corresponding process in insects, during which the legless larva or caterpillar becomes the winged fly or butterfly, living in a new habitat and on completely different foods, has marked experimental advantages because of the shortness of insect life cycles and the precision with which metabolic inputs, conversions and outputs can be characterized and controlled. For the past several years Dr. Levenbook and associates have been striving to answer the question of whether the proteins of the immature insect are reutilized in the new structures of the adult or are first broken down into their elements and reassembled. An exhaustive characterization of aldolase enzymes from muscle and from fat body (the insect homologue of the mammalian liver), chosen as model metabolic proteins, failed to reveal any chemical differences between the larval and adult blowfly, supporting the conclusion drawn earlier regarding a structural protein of larval and adult muscle.

With the virtual completion of the aldolase work, the Levenbook group has turned to control mechanisms of protein synthesis for a possible explanation of the fluctuations in protein content seen during development. Shortly before metamorphosis the total number of ribosomes per larva decreases due to destruction of fat body ribosomes and reaches a plateau that is maintained constant thereafter. Quite unexpectedly, however, it was found that a considerable fraction of the intact ribosomes remaining are free in the pupal blood, rather than intracellular, and that the number of these free ribosomes falls as adult development progresses. Some ribosomes doubly labeled in the larva later appear free in the pupal blood and still later in adult cells, with unchanged isotope ratio. These facts imply that ribosomes freed from histolysing larval tissue can be taken up intact by adult tissues.

All animal rhythms, whether metabolic or behavioral, circadian or short-cycle, can be presumed to be driven ultimately by neural or secretory pacemaker cells or networks. Most such pacemakers, even if identified, are inaccessible to direct experimental manipulation. A few, in which well defined sensory input is involved, can be controlled or at least influenced by external stimuli. Two such entrainable rhythms -- the flashing of certain oriental fireflies and a simple human motor behavior (voluntary finger-tapping) -- have been studied extensively by Dr. Buck and his group in an attempt to deduce some of the properties of the underlying oscillators. Two years ago a pacemaker with a very simple sawtoothed activity cycle, having the property of being completely reset in one step by artificial light pacer signals, was described from one species of firefly from New Guinea. A different and considerably more complex oscillator has now been discovered in a second New Guinea firefly. This oscillator runs when the firefly is not flashing and when it is being paced as well as during rhythmic free run flashing, and is resettable only partially and only within a narrow range close to the free run frequency. In certain zones of the cycle, pacer signals cause flash inhibition with accompanying cycle shortening, a behavior not explicable by analogy with the simple sawtooth relaxation oscillator. Whatever the ultimate nature of this pacemaker it appears to demand an excitable membrane that hyperpolarizes when spontaneously resetting, sensory resetting being, in contrast, only to the resting level.

Some years ago considerable work was done in LPB on human finger tapping. The existence of a predictive type of entrainment to acoustic signals (subject leading signal) was demonstrated at some frequencies of pacing, and ranges of variance, frequency response, frequency epicycles and long-period drift were established. Only a preliminary account of the work was published because the variability of the capacitative interval timer available and the necessity of recording by pen writer restricted time resolution to 10 msec. Dr. Laurens Mets has now resumed this work, using a crystal oscillator clock for timing and a frequency-modulated tape recorder permitting 1 msec time resolution and direct processing by digital computer. In some instances autocorrelation and time-series analyses of such free-run tapping data permit us to distinguish the variance due to the output part of the cycle from that due to the endogenous pacemaker (reference oscillator) in the brain. For example, it appears that the output leg in firefly pacemaking has a much lower variance than has the reference oscillator.

It is now generally agreed that most cell membranes contain a high proportion of lipid in the form of molecular bilayers and that entry and egress of solutes, whether active or passive, depend on such surfaces. The thermodynamics of chemically and physically defined lipid mono- and bilayers in relation to phase transitions, cholesterol interaction and the effects of steroid hormones have been studied rigorously by Dr. Norman Gershfeld and associates. These studies have developed a unified treatment of mono- and bilayers, showing that phospholipid in such layers behaves like the pure material in bulk. Recently it has been shown that addition of water to cholesterol-lecithin mixtures causes a separation into two phases, one the pure liquid-crystalline phospholipid and the other an anhydrous mixture. Cholesterol thus functions in maintaining the fluidity of the lipid membrane. Another new finding is that cell membranes of mycoplasma cells regulate the amount of cholesterol incorporated into their external membranes whereas isolated membranes cannot.

Dr. M. H. Gottlieb has been studying the properties of lamellar structures composed of phospholipid (lecithin) bilayers alternating with water. Last year he reported on the spacing of the lecithin sheets in relation to various ions, as defined by x-ray diffraction. He has now measured ion binding constants for the biologically important electrolytes to lecithin, finding no association of Na+ and K+, while Ca++, Li+, Mg++ and Ba++ (in descending order) are slightly bound. Dr. Gottlieb has also made a detailed study, using x-ray diffraction methods, of the transition between the two lamellar forms of lecithin-water mesophases, i.e., between the α-crystalline form in which the hydrocarbon chains of the lecithin are rigidly aligned and the liquid crystalline form in which the hydrocarbon chains have a liquid-like flexibility. The transition occurs over a temperature range of up to several degrees, depending on the water content of the mesophase. During the transition, mesophases of the two crystalline forms coexist in equilibrium, the liquid crystalline form having the higher water content. The computed thicknesses of the phospholipid bilayers in the α-crystalline form are greater than those of the bilayers in the coexisting liquid crystalline form, while the computed inter bilayer distances are the same in both coexisting forms.

All voluntary movement in animals is brought about by skeletal or ""striated" muscle in which the striations ("bands") divide the muscle cells transversely into visible segments or sarcomeres. Ultrastructural studies have shown that single muscle cells are composed of many attenuated fibrils and that the fibrils, in turn, contain two types of delicate filaments composed of either actin or myosin, the principal structural and kinetic proteins of muscle. The myofilaments are in longitudinal parallel array but do not run continuously through the length of the myofibril. Rather, they are divided into short segments, sarcomere by sarcomere, the thicker myosin filaments being anchored by their middles ("A band") and partly overlapping six of the thinner, actin filaments, which are anchored by their outer ends to the transverse "Z disc" structure. During muscular "contraction" muscle cells clearly shorten but about 15 years ago Andrew Huxley made a fundamental advance by proposing that this shortening did not involve actual contraction of the myofilaments but a sliding together of the interdigitating thick and thin filaments. Concurrently the electron microscope revealed minute projections from the myosin filaments which often seemed to form bridges to actin filaments. Much of the work on muscle during the past decade has concerned this "sliding filament model."

Dr. Podolsky and his associates have made several major advances in our understanding of "excitation-contraction coupling" and other aspects of muscle activity. In particular they have adduced evidence for the role of Ca++ as the trigger for excitation, its localization and regenerative release in the interfibrillar membrane system and its concentration relations to the development of force. A major investigation has focused on the kinetics of shortening in muscle fibers that have been activated at constant length, then allowed to shorten suddenly. Careful measurement and computer modeling of the transient changes during this process have enabled them to relate the shortening to the number, "reach", degree of independence and rate of attachment and detachment of the cross bridges.

In skinned muscle fibers the magnitude of the steady isometric force, and presumably of the spacing between the cross-bridges, can be controlled by varying the concentration of calcium ions in the bathing solution. At normal ionic strentgth (∿ 180 mM) the contraction velocity at a given relative load is independent of the calcium level. At lower ionic strengths the contraction velocity depends on calcium level, but this is interpreted to be due to the action of a second type of cross bridge which turns over, under these conditions, more slowly than normal. The contraction transients in skinned muscle fibers activated directly by calcium ions are similar to those in electrically stimulated intact fibers, which is evidence that the transients are slowed down proportionally when the temperature is lowered to 0°C. These results are inconsistent with the idea that the early part of the transient is due to a cross-bridge relaxation process and that the later parts are due to cross-bridge turnover. Rather, they indicate that the entire transient is due only to the making and breaking of cross-bridges.

Many medically vital constituents or products of cells, such as enzymes, hormones, viruses and antibodies, are protein macromolecules. One approach to elucidating the structure of such elements is to purify them to the point where they aggregate into crystalline arrays. If such crystals are large enough they can sometimes be analyzed by x-ray diffraction techniques. Smaller crystals are sometimes amenable to being "shadowed" with metal vapor and photographed in the electron microscope. By slicing the crystalline fabric in particular known planes, photographing the section after treatment to enhance electron-dense elements, optical intensification of the photograph, viewing the array along different axes and comparing the results with models, it is sometimes possible to deduce the size and structure of the unit molecule. Dr. Louis Labaw is the world's leading practitioner of this demanding technique. Among the molecules or molecular complexes whose structure he has elucidated are bacteriochlorophyll protein, thyroid ribosomes and polyribosomes, thyroglobulin, catalase, Bacillus thuringiensis protein and tobacco necrosis virus. His latest subject, human gamma globulin, is unusual in having a Y-shaped unit molecule.

There has been a steady growth of evidence that a cell is not merely a bag of biochemicals but that its functioning depends on definite 3-dimensional spatial relations between many of its constituents ("cytoarchitecture"). In particular it appears that activities such as protein synthesis may require transport and juxtaposition of macromolecular substrates and templates in precise and specific fashion. Using the flagellated protozoan Euglena Dr. Ellis Kempner and associates have been investigating cellular organization from two viewpoints: trying to define the complete input-output metabolic budget and observing the recovery of intracellular order after stratified by centrifugation. To date one impressive result of such disruption is the remarkable ability of the cell to survive the treatment and reestablish order. A second general finding is that all enzymes investigated are associated with discrete, visible particulates, rather than being in solution.

One of the few genetic diseases of man in which the defective molecule has not only been identified but its action explained is sickle cell anemia. Dr. Makio Murayama was a pioneer in establishing the chemical basis of this affliction. In the decade 1960-70, by a combination of experiment, intuition and molecular model building he had discovered the remarkable fact that the aggregation of the altered hemoglobin molecules deoxygenated in solution is promoted by raised temperature and reversed at low, shown that the molecules aggregate in parallel arrays confirmable by polarized light and the magnetic field and sometimes forming long microtubules visible in the electron microscope, shown the effects of propane and dilute urea in reversing the sickling of affected red blood cells, and developed a theory to explain the chaining of the modified hemoglobin. Today this work shows clinical promise, both diagnostically and therapeutically.

Serial No. NIAMDD-LPB-1
1. Physical Biology
2. Cellular Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Mechanism of Muscular Contraction

Previous Serial Number: Same

Principal Investigator: R. J. Podolsky

Other Investigators: Mark Schoenberg
A. C. Nolan
Marc D. Thames
Elizabeth W. Stephenson
Leepo C. Yu
Jagdish Gulati

Man Years (July 1972 through June 1973)

Total:	8.25
Professional:	7.25
Other:	1

Project Description:

Objectives:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.

Methods Employed:

1. Analysis of the motion and the X-ray diffraction pattern of both intact muscle fibers and "skinned" fiber segments under chemically controlled conditions.
2. Study of the electrical and chemical parameters involved in the activation of various types of muscle fibers.
3. Measurement of ionic fluxes across the internal membranes of skinned muscle fibers under various conditions.

Major Findings:

1. The propagation velocity of a mechanical impulse down the length of an activated muscle fiber is 10,000 cm/sec. This implies that the elastic strain is at least 40 A/half sarcomere.

2. In skinned muscle fibers the magnitude of the steady isometric force, and presumably of the spacing between the cross-bridges, can be controlled by varying the concentration of calcium ions in the bathing solution. At normal ionic strength (∿ 180 mM) the contraction velocity at a given relative load is independent of the calcium level. At lower ionic strengths the contraction velocity depends on calcium level, but this is interpreted to be due to the action of a second type of cross-bridge which turns over, under these conditions, more slowly than normal.
3. The contraction transients in skinned muscle fibers activated directly by calcium ions are similar to those in electrically stimulated intact fibers, which is evidence that the transients reflect cross-bridge behavior rather than activation processes. At 4°C the time course of the transient in the skinned fiber is half as fast as that in the intact fiber; all phases of the transient are slowed down proportionally when the temperature is lowered to 0°C. These results are inconsistent with the idea that the early part of the transient is due to a cross-bridge relaxation process and that the later parts are due to cross-bridge turnover. Rather, they indicate that the entire transient is due only to the making and breaking of cross-bridges.
4. Both the rate of calcium uptake and the calcium capacity of the sarcoplasmic reticulum are very sensitive to the level of ionized magnesium in the myofilament space. An increase of 0.1 mM Mg^{2+} stimulates net calcium uptake; a decrease of 0.1 mM induces net release.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the molecular mechanism of muscular contraction together with the chemistry of the activation process, can be useful in the rational handling of neuromuscular and cardiovascular disease.

Proposed Course of Project:

1. The influence of various agents (pCa, pH, ionic strength, metal ions, ATP) on the ability of muscle fibers to develop force and to shorten will be examined directly in skinned fiber segments.
2. The influence of these same agents on myofilament spacing and the cross-bridge configuration will be examined in skinned fibers by X-ray diffraction.
3. The effect of motion on the number of cross-bridges in intact, electrically stimulated muscle fibers will be studied by X-ray diffraction.
4. Movement of calcium and magnesium between the myofilament space and the sarcoplasmic reticulum will be studied by tracer methods.

Publications:

Podolsky, R. J., and Nolan, A. C.: Muscle contraction transients, cross-bridge kinetics, and the Fenn effect. Cold Spring Harbor Symp. 37: 661-668, 1972.

Podolsky, R. J.: Cross-bridge properties derived from muscle contraction transients. Proc. IV Int. Biophys. Congr., Acad. Sci. USSR (in press).

REPORTED IN PRESS - 1972

Ford, L. E., and Podolsky, R. J.: Calcium uptake and force development by skinned muscle fibers in EGTA buffered solutions. J. Physiol. 223: 1-19, 1972.

Ford, L. E., and Podolsky, R. J.: Intracellular calcium movements in skinned muscle fibers. J. Physiol. 223: 21-33, 1972.

Schoenberg, M., and Podolsky, R. J.: Length-force relation of calcium activated muscle fibers. Science 176: 52-54, 1972.

Serial No. NIAMDD-LPB-2
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Triggering of Bioluminescence. Sense of Rhythm.

Previous Serial Number: NIAMD-LPB-2

Principal Investigator: John B. Buck

Other Investigators: Drs. Laurens Mets and Kenneth Friedman, Mrs. Ruth Friedman (part-time), Mr. Charles H. Hanna

Cooperating Units: Mrs. Elisabeth M. Buck, Guest Worker; Dr. Frank A. Hanson, University of Maryland, UMBC, Catonsville, Maryland; Dr. James F. Case, University of California, Santa Barbara, California; Dr. Jean-Marie Bassot, Institut Oceanographique, Paris, France.

Man Years:

Total:	6-1/2
Professional:	5
Other:	1-1/2

Project Description:

Objectives:

To understand how action potentials in nerves are coupled to chemical light production in luminous organs. To understand how individual central nervous oscillators can become mutually entrained.

Methods Employed:

Standard electrophysiological and psychophysical. Special computer analyses.

Major Findings:

1. Though no success was achieved in developing a preparation to permit stimulating and recording from single firefly photocytes, experiments involving hemisectioning of the main nerve cord at various levels suggest that the pacemaker in the brain is connected with the light organ via through-conducting pathways that have no cross connections anterior to the terminal ganglionic mass (Dr. K. Friedman).

2. Analysis of records of entrainment of the New Guinea firefly *Luciola pupilla* to rhythmic pacing by external signals (cf. last year's report) was substantially completed. The data indicate that (a) the endogenous pacemaker continues to cycle independently of actual flashing or of concurrent pacing; (b) this species seems literally unable to flash repetitively outside the interflash period limits of 920 to 1030 msec (at 25°), this limitation being reflected in the narrow range of steady state pacing, the necessity for phase shifting by small aliquots and the broad range of inhibitory pacer signal timing; (c) period shortening or lengthenings due to pacer-firefly phase differences are independent and additive (Elisabeth and John Buck).

3. Preliminary statistical analyses of long serial records of free-run finger tapping indicate that (a) there are surprising individual differences in period variance, long-term frequency drift and degree of inter-cycle dependence, (b) some human endogenous motor rhythm differs from free-run flashing in fireflies in showing period-period interaction (whereas each firefly cycle seems to be independent), (c) there is clear indication of the existence in man of one (and probably two) reference oscillator in the brain, the variance in this oscillator being much lower than in the effector part of the tapping cycle. In fireflies, in contrast, variance in the flash-generating link is dominant (Laurens Mets).

Significance to NIAMDD Research:

The new data on rhythms and entrainment encourage the hope of eventually defining human pacemaking activity, separating timing from motor steps and understanding the ability to entrain to external pacing.

Proposed Course of Project:

No major changes anticipated.

Publications:

Buck, John: Bioluminescent behavior in *Renilla*. I. Colonial responses. *Biol. Bull.* 144: 19-42, 1973.

Halverson, Roger C., Case, James F., Buck, John, and Tiemann, Darwin: Control of luminescence in *Phengodid* beetles. *J. Insect Physiol.* In press.

Serial No. NIAMDD-LPB-3
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Physico-chemical properties of biological surfaces and related systems.

Previous Serial Number: NIAMD-LPB-20

Principal Investigator: N. L. Gershfeld

Other Investigators: K. Tajima

Cooperating Units: Department of Clinical Microbiology, Hadassah Medical School, Hebrew University, Jerusalem, Israel

Man Years (July 1972 through June 1973)

Total:	1.75
Professional:	1.75
Other:	0

Project Description:

Objectives:

(a) To measure the intermolecular energies in biological membranes and related structures.

(b) To evaluate the influence of intermolecular energies on the properties of cellular surfaces.

Methods Employed:

General techniques of surface chemistry have been developed to measure the thermodynamic properties of fluid surfaces. Differential scanning calorimetry has been applied to the analysis of phase relations in liquid crystals.

Major Findings:

1. The interaction of phospholipids with cholesterol has been studied by measuring the solubility of the former in molten cholesterol. From the relative lowering of the melting temperature of cholesterol it has been shown that the order of association is as follows, in decreasing order:

phosphatidyl choline > sphingomyelin > phosphatidyl ethanolamine > phosphatidyl serine $\approx$ phosphatidyl inositol. The interactions are due primarily to the polar moieties.

2. The complete phase diagram for cholesterol-dipalmitoyl lecithin anhydrous mixtures has been obtained. Preliminary evidence suggests that only one stoichiometric complex is formed at a ratio of cholesterol to DPL 4:1.

3. Upon addition of water to the lecithin cholesterol mixtures a separation of phases occurs, one phase consisting essentially of the pure liquid crystalline phospholipid, and the other of the anhydrous mixture.

4. The uptake of cholesterol by membrane preparations of mycoplasma indicates that the normal control mechanisms which limit the uptake of cholesterol in the whole cell is missing in the membrane preparation. For the membranes the apparent limit of cholesterol uptake is determined by the amount which go into the solution in which the membranes are suspended.

Significance to Biomedical Research and the Program of the Institute:

One of the least understood systems in membrane biology is the role cholesterol fills in the function of the membrane. The studies of the physical properties of cholesterol mixed with other lipids should permit the analysis of all equilibrium systems which contain cholesterol.

Proposed Course of Project:

The analysis of the effects produced by cholesterol in various physical systems will continue. In addition the cholesterol uptake studies by mycoplasma will be expanded to examine the role various phospholipids have in the uptake process. Both membranes and intact cells will be used.

Publications:

Gershfeld, N. L.: Thermodynamics and experimental methods for equilibrium studies with lipid monolayers. In Methods in Membrane Biology (in press).

Razin, S., Rottem, S., Hasin, M., and Gershfeld, N.: Binding of exogenous proteins and lipids to mycoplasma membranes. Ann. N. Y. Acad. Sci. (in press).

REPORTED IN PRESS - 1972

Pagano, R. E., and Gershfeld, N. L.: A millidyne film balance for measuring intermolecular energies in lipid films. J. Colloid Interface Sci. 41: 311-317, 1972.

Gershfeld, N. L., and Pagano, R. E.: The physical chemistry of lipid films at the air/water interface. I. Intermolecular energies in single component lipid films. J. Phys. Chem. 76: 1231-1237, 1972.

Pagano, R. E., and Gershfeld, N. L.: The physical chemistry of lipid films at the air/water interface. II. Binary lipid mixtures: the principles governing miscibility of lipids in surfaces. J. Phys. Chem. 76: 1238-1243, 1972.

Gershfeld, N. L., and Pagano, R. E.: The physical chemistry of lipid films at the air/water interface. III. The condensing effect of cholesterol; a critical examination of mixed film studies. J. Phys. Chem. 76: 1244-1249, 1972.

Serial No. NIAMDD-LPB-4
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Physical Chemistry

Previous Serial Number: NIAMD-LPB-5

Principal Investigator: M. H. Gottlieb

Other Investigators: None

Cooperating Units: Dr. E. D. Eanes, LBS/NIDR, Project No. NIDR-LBS-202b70

Man Years (July 1972 through June 1973)

Total: 1
Professional: 1
Other: 0

Project Description:

Objectives:

To understand the interactions between lipids, water and electrolytes, and the effects of such interactions on structures formed by the lipids.

Methods Employed:

X-ray diffraction, calorimetry, phase equilibria

Major Findings:

A method was developed to measure the binding constants of ions to phospholipids, such as lecithin, which swell, but do not dissolve, in water. This method is based on the distribution of electrolytes between the phospholipid-water phase and the purely aqueous phase coexisting with it. No significant binding between lecithin and Na^+ and K^+ ions was found, while Ca^{++}, Li^+, Mg^{++} and Ba^{++} ions (in descending order) were slightly bound. This sequence of ion binding agrees with that surmised from electrophoresis and film balance measurements. However, neither the sequence nor the magnitudes of the binding constants are in accord with the effects of electrolytes on the maximal solution contents of aqueous lecithin mesophases.

A detailed study, using X-ray diffraction methods, was made of the transition between the two lamellar forms of lecithin-water mesophases, i.e., between the α-crystalline form in which the hydrocarbon chains of the lecithin are rigidly aligned and the liquid crystalline form in which the hydrocarbon chains have a liquid-like flexibility. The transition occurs over a temperature range of up to several degrees, depending on the water content of the mesophase. During the transition, mesophases of the two crystalline forms coexist in equilibrium, the liquid crystalline form having the higher water content. The computed thicknesses of the phospholipid bilayers in the α-crystalline form are greater than those of the bilayers in the coexisting liquid crystalline form, while the computed interbilayer distances are the same in both coexisting forms.

Proposed Course of the Project:

If suitable techniques can be developed, X-ray diffraction studies on erythocyte membranes will be started (1) to attempt to detect crystalline transitions in the membranes similar to those reported on above for lecithin-water mixtures, and (2) to study the effects of cholesterol on the membrane structure. X-ray diffraction studies will be continued on the effects of electrolytes on the phospholipid bilayers of lecithin mesophases.

Significance to Biomedical Research and the Program of the Institute:

The properties of the bilayers in aqueous phospholipid mesophases should contribute to the understanding of the structure of cell membranes, particularly since the evidence is now overwhelming that the phospholipid exists as bilayers in cell membranes. X-ray diffraction studies of erythrocyte membranes should also contribute to the general understanding of membrane structures and possibly also to that of hemolytic anemias.

Publications:

Gottlieb, M. H., and Eanes, E. D.: Influence of electrolytes on the thicknesses of the phospholipid bilayers of lamellar lecithin mesophases. Biophys. J. 12: 1533-1548, 1972.

Serial No. NIAMDD-LPB-5
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies of metabolic activity in microorganisms.

Previous Serial Number: NIAMDD-LPB-21

Principal Investigator: Ellis S. Kempner

Other Investigators: Jay H. Miller

Cooperating Units: None

Man Years:

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

An understanding of the control of metabolic activity in unicellular organisms.

Methods Employed:

Culture of microorganisms in the presence of radioactive or other compounds; chemical and physical fractionation of the cellular materials by ultracentrifugation, chromatography and chemical extraction. Microscopic examination of cells by time-lapse video micrography.

Major Findings:

As with any move to a new laboratory, severe contamination problems arose in our axenic cultures of Euglena gracilis. The major contaminant was found to be a Penicillium species. Such molds are usually quite intractable because of their resistance to most compounds which kill other microorganisms. It was finally found that fluorodeoxyuridine, a powerful nucleotide analog, rapidly killed Penecillium while the Euglena were unaffected, presumably because of a permeability barrier. The only other contaminant, a yeast, is not inhibited by FUDR, but can be selectively destroyed by 8-azaguanine. This purine analog also does not inhibit Euglena. Growth rates of the Euglena cultures are not altered by either of these metabolic inhibitors.

After exposure to high centrifugal fields, the internal cytoplasm of Euglena is stratified due to separation of organelles of differing specific gravity. Upon return to normal culture conditions, the cells recover within several hours and are then indistinguishable from untreated controls. It is now well established that individual organelles are the sites of entire biochemical pathways; the integration of cellular metabolism must involve communication between these organelles. The spatial relationships between organelles can be temporarily altered by stratification, so that a study of cellular activities during this time should lead to an understanding of metabolic integration. During the recovery process, there is a 30 to 50% drop in cell population during the first five hours. Old experiments in a high-salt medium had never shown this loss of cells after centrifuging. Addition of compounds from the original recipe to the new minimal medium showed that the loss of cells after centrifugation was prevented by the presence of trace metals, specifically Na molybdate. The reasons for these surprising results are being examined.

Preliminary experiments of the rate of synthesis of protein and RNA, O_2 consumption and radiocarbon accumulation during the recovery process have been completed. Autoradiographic techniques are being developed to localize the incorporated radiocompounds in stratified cells.

It was previously reported that the dipeptide, arginyl glutamine, was identified in the "pool" extracts of Euglena; it is synthesized in cells grown on either glutamic acid or acetate as a sole carbon source as well as in cells grown photosynthetically. It is a metabolic intermediate showing the same rate of turnover as free amino acids. Unfortunately the attempts to specifically label the dipeptide with radio-arginine were blocked by cell permeability barriers. However, radio-glutamine shows promise of fulfilling cellular requirements for the dipeptide.

Significance to NIAMDD Research:

This work is aiding the development of a system for studying differentiated cellular structure in Euglena gracilis with the technical ease usual with bacteria. Using this system, new insight into the structure and function of cells is being gained.

Proposed Course of Project:

To study the metabolic relationships of synthetic processes in Euglena to the highly organized cellular structure which has already been elucidated.

Publications:

Skinner, H. Catherine W., Kempner, Ellis S., and Pak, Charles Y. C.: Preparation of the mineral phase of bone using ethylenediamine extraction. Calc. Tiss. Res. 10: 257-268, 1972.

Kempner, E. S. and Miller, J. H.: The molecular biology of *Euglena gracilis*. VII. Inorganic requirements for a minimal culture medium. *J. Protozool.* 19: 343-346, 1972.

Kempner, E. S. and Miller, J. H.: The molecular biology of *Euglena gracilis*. VIII. Glycitols and monosaccharides. *J. Protozool.* In press.

Serial No. NIAMDD-LPB-6
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Investigation of the macromolecular organization of living matter.

Previous Serial Number: NIAMDD-LPB-22

Principal Investigator: L. W. Labaw

Other Investigators: None

Cooperating Units: None

Man Years (July 1972 through June 1973)

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To gain information about the macromolecules that are essential constituents of living matter and to see how they are arranged in the structures they form. To study certain of these macromolecules, such as viruses and other proteins, in purified form after isolation from the living material. To improve the resolution of the electron microscope and to interpret the way in which images are formed near its limit of resolution.

Methods Employed:

Electron microscopy of microorganisms, cells and tissues in suspension or thinly sectioned. Physicochemical characterization of macromolecular components isolated from such material using electron microscope, x-ray diffraction, and similar established techniques.

Major Findings:

Crystals of phosphorylcholine-binding Fab-fragments from mouse myeloma (McPC603) immunoglobulin protein have been examined in the electron microscope. These crystals were grown by Dr. David R. Davies' group (LMB-NIAMDD) from protein supplied by Dr. Michael Potter (LCB-NCI) for their continuing study of the immunoglobulins by x-ray diffraction. The crystals studied in the EM were hexagonal prisms which a preliminary x-ray study had shown to belong to the $P6_3$ space group with unit cell dimensions of $\underline{a}$ = 162.6, $\underline{c}$ = 60.3 Å. The EM results confirmed the 162 Å cell dimension, but no periodicity along the prism axis could be found. The structure resembles a honeycomb since there are large open channels parallel to the prism axis, hexagonally arranged. The center-to-center spacing of these open channels is the 162 Å cell dimension so that the protein in the crystal lies along the three-fold axes, and the six-fold screw axes are the centers of the open channels. This has since been confirmed by a more complete x-ray diffraction analysis.

The lack of periodicity along the prism axis, when sections cut parallel to this axis are viewed in the EM, suggests that the Fab molecule (approximate dimensions 40 X 50 X 70 Å) is at an angle to a plane perpendicular to this prism axis. The more detailed x-ray diffraction analysis confirms this also.

Significance to Biomedical Research and the Program of the Institute:

The use of the electron microscope to determine the crystal structure of protein crystals is particularly important when applied to crystals which cannot be grown large enough for a more complete structure study by x-rays.

With increased emphasis on fine structure in electron microscopy, it is pertinent to examine the mechanism of image formation to define the conditions under which one can be assured that observed periodicities on images in the electron microscope have a direct spatial relationship with the object being examined when one is operating near the resolution limit of the instrument.

Proposed Course of Project:

To investigate other protein crystals in the electron microscope using replica, negative staining, and direct transmission techniques to determine their structures.

Publications:

Davies, D. R., Sarma, R., Labaw, L. W., Silverton, E., Segal, D., and Terry, W. D.: X-ray diffraction and electron microscope studies on a crystalline human immunoglobulin. Ann. N.Y. Acad. Sci. 190: 122-129, 1971.

REPORTED IN PRESS - 1972

Labaw, L. W. and Davies, D. R.: The molecular outline of human γG1 immunoglobulin from an EM study of crystals. J. Ultrastruct. Res. 40: 349-365, 1972.

Serial No. NIAMDD-LPB-7
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biochemical Changes during Development of Blowflies.

Previous Serial Number: NIAMD-LPB-3

Principal Investigator: L. Levenbook

Other Investigators: A. Bauer and S. Sridhara

Cooperating Units: Dr. P. S. Chen (University of Zurich, Switzerland)

Man Years:

Total:	2-3/4
Professional:	2-3/4
Other:	0

Project Description:

Objectives:

1. To study (a) Ribosomal and ribose nucleic acid metabolism during adult development of Blowflies, (b) the structure and function of calliphorin, (c) completion of comparison of larval and adult Blowfly muscle aldolases, (d) identification of paragonial factor from male Drosophila melanogaster.

Methods Employed:

Routine physical methods for the isolation of ribosomes, calliphorin and aldolases; routine chemical procedures for the study of isolated ribosomes and purified proteins.

Major Findings:

1. The total number of blowfly larval ribosomes decreases shortly before pupariation, and this reduced number remains constant throughout adult development. The concomitant reduction in total RNA is less than expected from the reduction in ribosomes. The reduction in ribosomes occurs almost exclusively as a result of destruction of fat boy ribosomes, the rRNA of which is degraded and stored in the fat body. During adult development there is a progressive increase followed by a decline in the number of larval ribosomes which are extracellular. Isotopic experiments indicate that at least some of these are taken up intact by the adult cells.

2. Experiments on calliphorin -- the major soluble protein of many insects -- are getting under way. Attempts are being made to improve the published method for preparation of pure calliphorin.

3. Larval and adult muscle blowfly aldolase have been shown to be chemically identical proteins. This project is now terminated.

4. Paragonial factor from Drosophila is a hormonal-like material which prevents mating and induces egg laying by virgin females. The present evidence indicates that the molecule is composed of equimolar amounts of glycerol, galactose and phosphoethanolamine; but there is no information as yet as to how these components are joined together.

Significance to NIAMDD Research:

The developing adult insect exhibits drastic tissue breakdown and extensive histogenesis; it thus serves as a model system for the study of ubiquitous cellular phenomena which may be obscure in man, but can be discerned in the insect by virtue of their occurring in an exaggerated form. This is well illustrated by the finding of blowfly extra-cellular ribosomes; if these were to occur at the site of (say) trauma in vertebrates, they would be difficult to find because of probable removal by the blood stream.

Proposed Course of Project:

Continuation of insect ribosomal project depends upon the availability of a suitable collaborator; detailed study of structure and function of calliphorin; elucidation of structure of the paragonial substance.

Publications: None.

Serial No. NIAMDD-LPB-8
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Molecular Mechanism of Human Red Cell Sickling and Unsickling

Previous Serial Number: NIAMD-LPB-4

Principal Investigator: Makio Murayama

Other Investigators: Dr. Alan S. Levine

Cooperating Units: None

Man Years:

Total:	1-1/2
Professional:	1-1/2
Other:	0

Project Description:

Objectives:

1. To find a molecular mechanism of "unsickling" of erythrocytes from sickle cell anemia patients.

2. To evaluate thermodynamic constants: the enthalpy change in the thermal (endothermic) aggregation of sickle cell hemoglobin (Hb S); the entropy change in the endothermic aggregation of Hb S; and the volume of activation in the endothermic aggregation of Hb S.

3. To investigate the mode of action of urea in unsickling.

4. To examine the specificity of the oligodynamic action of urea.

5. To investigate the effect carbamylation of amino 1 of the β-chain by cyanate (or carbamyl phosphate) on sickling.

Methods Employed:

Aggregation-deaggregation of sickle cell hemoglobin by pressure, temperature, and millimolar urea. Structural details of sickling and unsickling by differential interference optical (Nomarski optics) microscopy and time lapse cinephotomicrography.

Major Findings:

Confirmed most of the findings reported last year. Desickling property of an extract of Fagara xantheroides (an African chewing stick) has been observed several times.

Significance to NIAMDD Research:

Sickle cell anemia is a hereditary, non-infectious hemolytic anemia, hence significant to the NIAMDD mission. My theory has been applied at the bed side with success here as well as in Africa (oral urea therapy and oral urea prophylaxis).

Proposed Course of Project:

The extract of Fagara xantheroides will be investigated further. Initial isolation of the active ingredient(s) and then identification and the usual process of working out the structure and so forth. Then knowing the structure that might cause desickling, we might figure out the process of sickling.

Honors:

Member, Editorial Advisory Board, Foundation for Research and Education -- Sickle Cell Disease, 423-431 W. 120th Street, New York, New York, effective September, 1972.

Publications:

Murayama, M.: Thermal (or endothermic) aggregation of sickle cell hemoglobin (Hb S) during sickling. In Brewer, G. J. (Ed.): Hemoglobin and Red Cell Structure and Function. New York, N.Y., Plenum Press, 1973, pp. 243-251.

Author: Murayama, M. and Nalbandian, R. N.: Sickle Cell Hemoglobin: Molecule to Man. Boston, Little, Brown and Co. In press.

Murayama, M.: The chemical and the three-dimensional structure of human hemoglobin. In Murayama, M. and Nalbandian, R. N. (Authors): Sickle Cell Hemoglobin: Molecule to Man. Boston, Mass., Little, Brown, and Co. In press.

Murayama, M: Bioenergetics of human red cell sickling. In Murayama, M. and Nalbandian, R. N. (Authors): Sickle Cell Hemoglobin: Molecule to Man. Boston, Mass., Little, Brown and Co. In press.

Murayama, M.: Molecular hypothesis for the mechanism of sickling. In Murayama, M. and Nalbandian, R. N. (Authors): Sickle Cell Hemoglobin: Molecule to Man. Boston, Mass., Little, Brown and Co. In press.

Murayama, M.: My individual science. Proceedings of 20th Annual Blood Symposium: Sickle Cell Hemoglobin. In press.

ANNUAL REPORT SUMMARY
LABORATORY OF BIOPHYSICAL CHEMISTRY

The chief interest of this laboratory has been the study of the relationship between structure and function of biological macromolecules, especially those proteins involved in muscular contraction, blood coagulation, and in carrying oxygen. Advantage is also taken when pathological conditions offer insight into the problem under investigation. Special attention is paid to the interaction of these proteins with their environments, such as water, certain ions, and ATP.

HEMOGLOBIN: Our studies on the binding of both H^+ and Na^+ to 2,3-Diphosphoglyceric acid (DPG) and the studies in other laboratories on the effect of DPG on (1) oxygen binding and (2) the Bohr effect provide data for an attempt to rationalize the action of DPG and hemoglobin in terms of our model.

The energetics of the reaction of hemoglobin with oxygen and protons has been rationalized in terms of the available data. In our analysis, the interaction energy the oxygenated hemoglobin tetramer is at a higher potential energy state than the deoxy tetramer. Under physiological conditions, the oxygenation reaction furnishes the energy of interaction. (Saroff)

The number of SH groups per tetramer of mammalian hemoglobin varies from two to six. The role of these SH groups in the action of hemoglobin is being studied. (Saroff and Irreverre)

A theoretical model for the gelation of deoxy HbS has been developed which appears to account for essentially all reliable reported data on the temperature and concentration dependence of gelation. Observations in other laboratories, confirmed by our own viscosity measurements, indicate that the formation of low order aggregates is not enhanced by deoxygenation. This heretofore puzzling observation is accounted for by the aforementioned model. Since gelation is the cause of erythrocyte sickling in sickle-cell anemia, it is hoped that a knowledge of the molecular basis of gelation will suggest appropriate chemotherapy for the remission or prevention of sickle-cell crises. (Minton)

MUSCLE AND CONTRACTILE PROTEINS: (1) MUSCLE: In a variety of cells, aging and death are correlated with loss of serotonin sensitivity and appearance of ATP sensitivity. Both living and dead cells decreased in volume on contracting. (Kominz, Wolford and Lander)

Collagenase treatment in the presence of Ca^{++} released damaged cells from chicken gizzard, but treatment with proteolytic enzymes in Ca^{++}-free solution released intact, living smooth muscle. (Kominz and Stewart)

The dynamic length-tension interaction is a clear measure of the energy-utilizing process of contraction; if the energy-producing process could be simultaneously monitored, the elusive coupling between the two processes could be studied. For such studies, a servoforce-balance accelerometer was modified into a highly isometric force transducer, comparable in sensitivity to other transducers currently in use with very small muscle fibers. (Goldstein, Kominz and Tsukui)

(2) TUBULIN: Tubulin, the protein of microtubules, is also involved in movement as in flagellum and the mitotic spindle. To a great extent, this protein resembles actin. Our purpose is to see how far this similarity goes. Starting from this point of view, tubulin was isolated from pig brain and various of its properties studied. It was found that the SH reagent NEM influenced the colchicine binding capacity of this protein. Similar to actin, the presence of tubulin increased the hydrolysis of the nucleotides GTP and ATP under sonication. The amino acid analysis of our tubulin preparation appears similar to an actin preparation which is known to be contaminated with tropomyosin or troponin.

FIBRINOGEN: It has been found that salmon plasma contains an exceedingly powerful "plasmin-like" protease capable of destroying the fibrinogen (or fibrin) even when the plasma is stored at -20°C. We have been able to overcome this obstacle by working in the cold at all times and utilizing fresh plasmas, and by inhibiting this protease by preincubation of the plasma at 2°C with large quantities of DFP.

With the above problem circumvented, we have been able to show that the action of (bovine) thrombin on highly purified fractions of salmon fibrinogen resulted in the release of two fibrinopeptides and that the linkage hydrolyzed on the fibrinogen was between arginine and glycine. This is the same as has been shown for all species, even though some 400-500 million years separate man from fish.

Our experiments have shown that two cross-linking enzyme systems exist in salmon plasma. This plasma contains the classical "thrombin" or "thrombin-like" activated enzyme which forms cross-links between the fibrin monomers, the Laki-Lorand Factor or Factor XIII. This enzyme cross-links the γ chains to give a $\gamma\gamma$-dimer and either the α- or β-chains to give a higher molecular weight aggregate.

In addition to the above mentioned cross-linking enzyme system, we have clearly demonstrated the existence in salmon plasma of a second cross-linking enzyme of the transglutaminase variety, such as that reported by Folk and Chung, to exist in pig liver. This enzyme can cross-link all the fibrinogen chains into a high molecular weight complex, no thrombin being required. It will also cross-link two of the chains of fibrin, the α- and γ- into a similar high molecular weight complex, (there is no $\gamma\gamma$-dimer as found with a Factor XIII), but appears to leave the β-chain untouched. (Gladner, Murtaugh)

The succinylated derivatives of both bovine fibrinogen and fibrin revealed that the α-chain of these modified proteins appears to be susceptible to further hydrolysis by thrombin. This appears to be the first instance where thrombin attack has gone to another area besides the heretofore known four bonds of arginine and glycine. (Gladner, Lewis, Murtaugh)

CLOT RETRACTION: Our studies concerned the investigation of whether actin interacts with the fibrin network. Using rabbit actin and bovine fibrinogen, it was found that fibrinogen does not, but fibrin does, interact with F-actin. Apparently in this combination, actin undergoes some structural change because it readily releases its bound nucleotide. In spite of the undoubted presence of actin in the mixed clot (fibrin + actin), electron microscopy reveals only the presence of fibrin strands suggesting that actin somehow blended into the fibrin network. Oriented, dried fibers have been prepared from the mixed clot and these will be examined by X-ray diffraction to see if the so-called actin diffraction pattern can be discerned.

Clot retraction is a vital, last step in blood coagulation. If it turns out that indeed actin and myosin-like proteins are involved in this process, then clarification of clot retraction will lead to a better understanding of muscular contraction as well. (Laki)

MODIFICATION OF PROTEINS: Studies of the structural features of proteins, and the elucidation of the structures of unusual amino acids, peptides and metabolites are expected to contribute to our knowledge and understanding of living systems including man.

We are studying the cyanate reaction for the determination of end groups in proteins with particular emphasis on revealing the possibility of occurrence of mobile end groups in proteins. The most sensitive step in the reaction appears to be the one which generates the pyrrolidone carboxylic acid derivatives when the cyanate reaction product is cyclized to form the hydantoin. (Irreverre and Saroff)

In connection with the role of the SH groups in proteins, the kinetics of the acyl shift reactions in glutathione and N-formyl cysteine have been determined. (Simpson)

Serial No. NIAMDD LBC-1
1. Biophysical Chemistry
2. Bioenergetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Basic mechanisms of muscular contraction.

Previous Serial Number: LBC-1

Principal Investigator: D. R. Kominz

Other Investigators: Dr. Ryoko Tsukui, Visiting Associate, Univ. of Tokyo, Mr. Clarence Israel

Outside Collaborators: Dr. Ute Stewart, Wurzburg, Germany; Dr. Seth Goldstein, BEIB, DRS; Mrs. Nancy Wolford and Miss Marilyn Lander, LVD, NIAID; Dr. Evan Eisenberg, LB, NHLI

Man Years
Total: 3.5
Professional: 2.0
Other: 1.5

Project Description:

Objectives: To advance the understanding of contractility and its regulation.

Methods: In general, studying the physical properties and functional activities of glycerinated muscle fibers, suspensions of myofibrils and isolated single cells, and solutions of purified contractile proteins. Specifically, 1) utilizing a sensitive transducer of new design to measure simultaneously tension, length and transmitted light in response to rectilinear or sinusoidal length changes; 2) utilizing a multichannel analyzer in multiscaling mode to record these three measurements simultaneously in digital format; 3) utilizing the multichannel analyzer in pulse height analysis mode to study length and volume changes of various single cell preparations, with kinetic studies facilitated by a rapid storage tape buffer; and 4) utilizing ultracentrifuge, gel electrophoresis, spectrophotometer-pH stat, radioactive labelling and other techniques as required.

Major Findings: 1. In a variety of cells, aging and death were correlated with loss of serotonin sensitivity and appearance of ATP sensitivity. Both living and dead cells decreased in volume on contracting. (Kominz, Wolford and Lander)

2. Collagenase treatment in the presence of Ca^{++} released damaged cells from chicken gizzard, but treatment with proteolytic enzymes in Ca^{++}-free solution released intact, living smooth muscle. (Kominz and Stewart)

3. A servoforce-balance accelerometer was modified into a highly isometric force transducer, comparable in sensitivity to other transducers currently in use with very small muscle fibers. (Goldstein, Kominz and Tsukui)

4. Increasing pH from 6.5 to 7.7 caused a large increase in the elastic modulus of EGTA-relaxed glycerinated rabbit psoas fibers. (Tsukui and Kominz)

5. Intermediate complexes could be clearly distinguished from the separate components of troponin in the ultracentrifuge. (Eisenberg, Kominz and Tsukui)

Projects Proposed: 1. Single living smooth muscle cells will be prepared. Dose-response curves to pharmacological agents will be attempted, and if successful will be compared to those obtained with the intact tissue.

2. Preparation of glycerinated fibers with reproducible tension responses and the ability to perform work will be sought. Length, tension and optical properties will be simultaneously monitored under varied conditions of rest, rigor and contraction. Modifications produced by selective extraction and by adding antibodies specific for various constituent contractile proteins will be examined. Following the experiment the specimen will be fixed in situ and examined by electron microscope.

Significance to Bio-Medical Research and the Programs of the Institute:

1. There is a practical need to measure in some way the pharmacological response of small clusters of sensitive cells not amenable to usual procedures, such as bronchiolar smooth muscle. Work planned on the preparation of living smooth muscle cells and measurement of their response is directly related to this need. It will probably not even be necessary to separate different cell types preparatively, since the instrumentation can analyze groups of cells separately on the basis of their size.

2. The dynamic length-tension interaction is a clear measure of the energy-utilizing process of contraction; if the energy-producing process could be simultaneously monitored, the elusive coupling between the two processes could be studied.

Honors and Awards: None

Publications: None

Serial No. NIAMDD LBC-2
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Intermolecular interactions in physical biochemistry

Previous Serial No.: NIAMDD LBC-4

Principal Investigator: Allen P. Minton

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: During the past year an attempt has been made to interpret the phenomenon known as "gelation" in solutions of deoxygenated sickle-cell hemoglobin (deoxy HbS) in terms of intermolecular interactions and the structure of hemoglobin and its aggregates.

Methods: Experiments were performed using methods of precision capillary viscometry. Theoretical studies employed methods of classical and statistical thermodynamics.

Major Findings and Proposed Course of Studies: A theoretical model for the gelation of deoxy HbS has been developed which appears to account for essentially all reliable reported data on the temperature and concentration dependence of gelation. Observations in other laboratories, confirmed by our own viscosity measurements, indicate that the formation of low order aggregates is not enhanced by deoxygenation. This heretofore puzzling observation is accounted for by the aforementioned model.

We propose to extend the model to account for the effects of pH, ionic strength and the presence of small molecules upon the gelation process. In addition experiments are planned to characterize the temperature and concentration dependence of the gelation process in greater detail and with greater precision than currently provided by the literature.

Significance to Biomedical Research and the Programs of the Institute: The theoretical model developed here brings us closer towards the complete understanding of the molecular basis of the gelation of HbS. Since gelation is the cause of erythrocyte sickling in sickle-cell anemia, it is hoped that a knowledge of the molecular basis of gelation will suggest appropriate chemotherapy for the remission or prevention of sickle-cell crises.

Honors & Awards: None

Publications:

Reisler, E., Eisenberg, H., and Minton, Allen P.: The temperature and density dependence of the refractive index of pure liquids. J. Chem. Soc., Faraday Trans. II 68, 1001 (1972).

Minton, Allen P.: Models for the gelling behavior of binary mixtures of hemoglobin variants. J. Mol. Biol. 75, 559 (1973).

Serial No. NIAMDD LBC-3
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The elucidation of the structure and interactions of biologically important macromolecules.

Previous Serial Number: LBC-2

Principal Investigator: H. A. Saroff

Other Investigators: Dr. R. B. Simpson, E. R. Mitchell and H. L. Wolff

Cooperating Units: Dr. Arthur Spector, Univ. of Iowa Clinical Research Center, Iowa City, Iowa; Dr. J. Preer, Federal City College, Washington, D. C.

Man Years:
Total: 4½
Professional: 4
Other: ½

Project Description:

Objectives: The main objective of this laboratory is an elucidation of the structure of naturally occurring macromolecules based on the methods of physical chemistry. Macromolecules are studied primarily in solution and the emphasis is on the accumulation of data on the state of the molecules with respect to environment in solution. Gross features of structures as well as fine detail, may be observed in the study of macromolecules in solution.

Methods: At present, we are concerned with methods of observing macromolecules in solution. In our studies aimed at finer details of protein structure, the methods used involve the measurements of the binding of small molecules or ions to specific sites on the protein molecule, nuclear magnetic resonance (on model compounds, so far), optical rotatory dispersion measurements and other spectral techniques. For other properties of the protein molecules, we employ the ultracentrifugal, light scattering, electrophoretic and diffusion techniques.

Major Findings and Proposed Course of Studies: Our studies on the binding of both H^+ and Na^+ to 2,3-Diphosphoglyceric acid (DPG) and the studies in other laboratories on the effect of DPG on (1) oxygen binding and (2) the Bohr effect provide data for an attempt to rationalize the action of DPG and hemoglobin in terms of our model.

The energetics of the reaction of hemoglobin with oxygen and protons has been rationalized in terms of the available data. The following summarizies our conclusions.

1. With respect to the interaction energy the oxygenated hemoglobin tetramer is at a higher potential energy state than the deoxy tetramer.

2. Under physiological conditions, the oxygenation reaction furnishes the energy of interaction.

The number of SH groups per tetramer of mammalian hemoglobin varies from two to six. The role of these SH groups in the action of hemoglobin is being studied. (Saroff and Irreverre)

In connection with the role of the SH groups in proteins, the kinetics of the acyl shift reactions in glutathione and N-formyl cysteine have been determined. (Simpson)

Studies are being continued on the use of computational models for the binding of ions to clusters of ionizable groups in model compounds. (Preer, Saroff)

Attempts to crystallize serum albumin and a mercury derivative suitable for X-ray crystallography continue. (Simpson)

Studies on aqueous solutions of fatty acids are being continued with Arthur Spector of the University of Iowa.

Significance to Biomedical Research and the Programs of the Institute:

Our emphasis is on the relationship between chemical properties and the structure of macromolecules. Once these properties are properly related, the attack on the relationship between structure and function can proceed on a solid basis. The proteins and macromolecules studied are either enzymes or those of interest in biological systems. Our systems are highly controlled with reference to the conditions of the protein environment and the bio-med-medical significance of our findings derive from extropolation to the biological system.

Honors and Awards: None

Publications:

Saroff, H. A.: The action of hemoglobin. Cooperative and Bohr effects. J. of Phy. Chem. 76, 1597 (1972).

Saroff, H. A.: Action of hemoglobin. The energy of interaction. Biopolymers 12, 599 (1973).

Serial No. NIAMDD LBC-4
1. Biophysical Chemistry
2. Physical Biochemistry
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Proteins, enzymes and peptides involved in blood coagulation and inflammation.

Previous Serial No.: NIAMD LBC-4

Principal Investigator: Jules A. Gladner

Other Investigators: Mrs. Patricia A. Murtaugh

Cooperating Units: Dr. John Halver, Chief, Fish Nutrition Lab., Fish and Wildlife Service, Dept. of Interior, Cook, Washington; Dr. Marc S. Lewis, NEI, NIH; Dr. John C. Houck, Children's Hospital, Washington, DC; and Dr. Claude B. Klee, NIAMDD-LBP.

Man Years:
Total: 4
Professional: 4

Project Description:

Objectives: As previously described, our major investigations involve the protein-protein interactions concerned with two vital life processes. These mechanisms, which appear unrelated, are the intricate molecular aspects of blood coagulation, especially as applied to the broad aspects of the thrombin-catalyzed conversion of fibrinogen to fibrin plus the ensuing reactions, such as fibrin cross-linking. The second of these investigations concerns the action of anti-inflammatory drugs to induce proteolytic enzymes which are highly specific in their actions, "limited proteases", and appear to play a major role in the biochemistry of the inflammatory response. Thus, the two apparently physiologically unrelated mechanisms are similar in that their effects are brought about via highly specific protein-protein interactions.

Methods: Methods employed consisted of protein-enzyme fractionation, purification, characterization and modification. Where needed, methods of physical biochemistry and analytical chemistry were utilized, such as but not limited to, SDS gel electrophoresis and automatic amino acid analysis. Physiological techniques were utilized to examine the biological activity of various peptides. As applicable, new techniques were developed.

Major Findings: Thrombin conversion of fibrinogen to fibrin and cross-linking of fibrin monomers. Our major attention has been addressed to this process in the lower species, especially fresh water lamprey eel and Columbia River salmon. Much progress has been made utilizing the fresh plasma of Columbia River salmon collected at the beginning of the spawning season (mid-fall) when the fish come up the river. The fresh anti-coagulated plasma was rapidly frozen and shipped to Bethesda. The following results have been obtained:

We have now been able to discern why other investigators were unable to obtain clottable salmon fibrinogens of high purity. It has been found that salmon plasma contains an exceedingly powerful "plasmin-like" protease capable of destroying the fibrinogen (or fibrin) even when the plasma is stored at -20°C. We have been able to overcome this obstacle by (1) working rapidly and in the cold at all times and utilizing fresh plasmas; (2) inhibiting this protease by preincubation of the plasma at 2°C with large quantities of DFP. It is our feeling that this "plasmin-like" enzyme could have definite therapeutic benefits and efforts will be made in the future to work out isolation and purification techniques.

With the above problem circumvented, we have been able to show that the action of (bovine) thrombin on highly purified fractions of salmon fibrinogen resulted in the release of two fibrinopeptides and that the linkage hydrolyzed on the fibrinogen was between arginine and glycine. This is the same as has been shown for all species, even though some 400-500 million years separate man from fish.

Cross-linkage experiments proved rewarding and interesting: Our experiments have shown that two cross-linking enzyme systems exist in salmon plasma. This plasma contains the classical "thrombin" or "thrombin-like" activated enzyme which forms cross-links between the fibrin monomers, the Laki-Lorand Factor or Factor XIII. This enzyme cross-links the γ chains to give a γγ-dimer and either the α- or β-chains to give a higher molecular weight aggregate. To date, we have not been able to discern which of the two chains gives the latter results, although in the human and bovine species, it has been shown to be the α-chain. Incorporating experiments now planned will clarify these results.

In addition to the above mentioned cross-linking enzyme system, we have clearly demonstrated the existence in salmon plasma of a second cross-linking enzyme of the transglutaminase variety, such as that reported by Folk and Chung, to exist in pig liver. This enzyme can cross-link all the fibrinogen chains into a high molecular weight complex, no thrombin being required. It will also cross-link 2 of the chains of fibrin, the α- and γ- into a similar high molecular weight complex, (there is no γγ-dimer as found with Factor XIII), but appears to leave the β-chain untouched. This transglutaminase can also be inhibited by prior treatment of the salmon plasma with DFP as previously shown to inhibit the "plasmin-like" protease. At present, we do not know if (1) the DFP inhibits the enzyme directly or (2) the DFP inhibits the activation of the enzyme to its active form or (3) if the enzyme is accelerated during the course of the salmon plasma fractionation for fibrinogen and the DFP acts at this step. All these postulates are under experimentation. (Murtaugh, Gladner, Halver).

Modified fibrinogens and fibrins. The succinylated derivatives of both bovine fibrinogen and fibrin are still under study as to their kinetic-molecular aspects. Our studies also show that the α-chain of these modified proteins appears to be susceptible to further hydrolysis by thrombin. This appears to be the first instance where thrombin attack has gone to another area besides the heretofore known four bonds of arginine and glycine. Other succinylated proteins, insulin, human serum albumin and hemoglobin are also being investigated as to the possibility of further understanding the unique specificity of thrombin. (Gladner, Lewis, Murtaugh).

Active site of histidine ammonia-lyase. Employing radioactive tracers, enzymatic degradation, column chromatography, peptide mapping and amino acid analysis, the active site of this enzyme was isolated and characterized. (Klee, Gladner).

Significance to biomedical research and the program of the Institute: In addition to that which has been mentioned in previous reports, the major addition to this report is the discovery in salmon plasma of this extremely active "plasmin-like" protease which has the capability to rapidly destroy fibrinogen, and especially fibrin. The isolation of this fibrin-lysing-like enzyme may have a real possible therapeutic value.

Proposed course of project: Investigations are anticipated to proceed along the general lines outlined in the bulk of this report. Emphasis will be placed on the isolation of the "plasmin-like" protease and the cross-linking enzymes of salmon plasma.

Honors and awards: None.

Publications:

1. Klee, C. B. and Gladner, J. A.: Isolation of a cysteine-peptide at the active site of histidine ammonia-lyase. *J. Biol. Chem*. 247: 8051-8057, 1972.

Serial No. NIAMDD LBC-5
1. Biophysical Chemistry
2. Physical Biochemistry
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biochemical studies on physiologically important micro and macromolecules in living systems.

Previous Serial No.: NIAMD LBC-3

Principal Investigator: Filadelfo Irreverre

Technical Assistance: Mr. Edwin Wilson

Cooperating Units: Dr. J. L. Hartwell, DD, NCI
Dr. H. Tabor, LBP, NIAMDD
Dr. C. W. Tabor, LBP, NIAMDD

Man Years
Total: 2
Professional: 2

Project Description:

Objectives: Living systems are made up of rigid molecular structures and circulating fluids carrying a myriad of macro and micromolecules. It is of interest to direct studies on (1) the nature of some of the unknown nitrogenous molecules, and (2) structural features of some proteins such as the influence of various residues with neighboring groups.

Methods: Techniques in chemistry and biochemistry have been routinely employed, such as paper and column chromatography, automated amino acid analysis, thin layer chromatography, gel separation and filtration, paper and gel electrophoresis, UV, infrared and mass spectrometry, NMR, polarimetry, ultracentrifugation, radioisotopic techniques and enzymatic analysis. When necessary, development of new procedures of isolation, detection or micro-assay has been undertaken.

Major Findings: We are studying the cyanate reaction for the determination of end groups in proteins with particular emphasis on revealing the possibility of occurrence of mobile end groups in proteins. The most sensitive step in the reaction appears to be the one which generates the pyrrolidone carboxylic acid derivatives when the cyanate reaction product is cyclized to form the hydantoin. (Irreverre and Saroff).

Developed a quantitative determination of aliphatic diamines and polyamines by an automated liquid chromatographic procedure. The method can be used for a variety of biological materials, including urine, animal tissues, and bacterial extracts. (H. Tabor, C. W. Tabor and F. Irreverre).

With materials furnished by the Drug Development Branch, NCI, we continued to examine various plant extracts looking for the presence of unusual amino acids.

Significance to bio-medical research and the program of the Institute: Studies of the structural features of proteins, and the elucidation of the structures of unusual amino acids, peptides and metabolites will contribute to our knowledge and understanding of living systems including man.

Proposed course of project: Biochemical studies of physiologically interesting micro and macromolecules in living systems will continue. Special emphasis will be given to uncovering the possible occurrence of mobile end groups in proteins.

Honors and awards: None.

Publications:

1. Tabor, H., Tabor, C. W. and Irreverre, F.: Quantitative determination of aliphatic diamines and polyamines by an automated liquid chromatographic procedure. Anal. Biochem. (In press).

Serial No. NIAMDD LBC-6
1. Biophysical Chemistry
2. Physical Biochemistry
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: A study of clot retraction and microtubular proteins.

Previous Serial No.: None

Principal Investigators: K. Laki
Dr. Maria Straub, Visiting Associate

Technical Assistance: None

Man Years
Total: 1-3/4
Professional: 1-3/4

Project Description:

Objectives: Our objective in the current project is to begin a systematic study of the process of clot retraction. This is a vital stage in the process of blood coagulation. According to current views, the actin and myosin-like proteins of the disintegrating platelets are involved. Clot retraction then imitates muscular contraction.

Tubulin, the protein of microtubules, is also involved in movement as in flagellum and the mitotic spindle. To a great extent, this protein resembles actin. Our purpose is to see how far this similarity goes.

Methods: Methods employed involve enzyme, protein isolation, purification, the tools of protein chemistry such as ultracentrifuge, gel electrophoresis, X-ray diffraction, electron microscopy, and amino acid analysis. Enzymatic degradations, radioisotopic techniques, thin layer chromatography and others are also utilized.

Major Findings: Our studies concerned the investigation of whether actin interacts with the fibrin network. Using rabbit actin and bovine fibrinogen, it was found that fibrinogen does not, but fibrin does, interact with F-actin. Apparently in this combination, actin undergoes some structural change because it readily releases its bound nucleotide. In spite of the undoubted presence of actin in the mixed clot (fibrin + actin), electron microscopy reveals only the presence of fibrin strands suggesting that actin somehow blended into the fibrin network. Oriented, dried fibers have been prepared from the mixed clot and these will be examined by X-ray diffraction to see if the so-called actin diffraction pattern can be discerned.

Microtubular proteins resemble to a great extent the muscle protein, actin. Starting from this point of view, tubulin was isolated from pig brain and various of its properties studied. It was found that the SH reagent NEM influenced the colchicine binding capacity of this protein. Similar to actin, the presence of tubulin increased the hydrolysis of the nucleotides GTP and ATP under sonication. The amino acid analysis of our tubulin preparation appears similar to an actin preparation which is known to be contaminated with tropomyosin or troponin.

Significance to biomedical research and the program of the Institute: Clot retraction is a vital, last step in blood coagulation. If it turns out that indeed actin and myosin-like proteins are involved in this process, then clarification of clot retraction will lead to a better understanding of muscular contraction as well.

Microtubules are instrumental in the process of cell division, in the maintenance of cell shape, and judging from their quantity in brain cells, they have an important, as yet not understood, role in the functioning of the brain.

Proposed course of project: We are attempting to see whether tropomyosin or troponin-like proteins can be isolated from our tubulin preparations. During the course of this project, we intend to study the interaction of other muscle proteins such as myosin, tropomyosin and troponin to fibrinogen.

Honors and Awards: None.

Publications:

1. Laki, K.: Clotting systems of human blood and their significance. J. Hung. Chem. 6, 27: 290-296, 1972.

2. Laki, K. and Wilson, E.: The action of hydroxylamine on myosin. Physiol. Chem. & Physics 4: 263-270, 1972.

Serial No. NIAMDD LBC-7
1. Biophysical Chemistry
2. Physical Biochemistry
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The action of thrombin on fibrinogen.

Previous Serial No.: NIAMD LBC-7

Principal Investigator: A. J. Osbahr

Man Years
    Total: 1
    Professional: 1

Project Description:

Objectives: The chemical structure and the physiological role of the peptides released from fibrinogen were investigated. Modification of thrombin, fibrinogen and the peptides derived from them was studied to ascertain the site of enzymatic and physiological activity. Of major interest is the investigation of the structural participation of the peptides from fibrinogen in blood clotting mechanism, hemostasis, vasoconstriction and pulmonary arterial blood pressure. Also of interest is the etiology of respiratory distress syndrome and diseases associated with high blood pressures. The presence of peptides from fibrinogen due to intervascular clotting may well explain many systemic manifestations associated with these diseases. We hope to demonstrate the presence of fibrinopeptides as a possible agent for such vasoconstriction.

Methods: The methods applied to study these systems were those of structure determination and sequence analysis with the special application of techniques to evaluate homogeneity and purity of the systems under investigation. More specialized techniques of physiology were utilized when needed to study the biological role of the products of blood coagulation.

Major Findings: The modification of fibrinogen. The methylation of fibrinogen resulted in a polymerized formation resembling a fibrin clot. (Further work on characterizing this is in progress). The methylated fibrinogen was insoluble in 0.1 M ammonium acetate, 0.3 potassium chloride and 0.3 M ammonium bicarbonate pH 8.0. The methylated fibrinogen was soluble in 6 M urea and 2 M acetic acid and polymerization was reversible upon dialyzing out the 6 M urea. The methylated fibrinogen was reduced in 6 M urea with 2-mercaptoethanol and alkylated with iodoacetamide. The reduced and alkylated fibrinogen was applied to a G-100 Sephadex column equilibrated in 6 M urea with 0.3 M ammonium bicarbonate buffer pH 8.0. One defined peak and three overlapping peaks were separated. The overlapping peaks were rechromatographed on DEAE-cellulose equilibrated in 6 M urea and 0.3 M ammonium bicarbonate pH 7.8 with a linear gradient of sodium chloride 0.05 M to 1 M. Some separation was achieved and at least one more peak could be separated. Additional ion exchange chromatography will be necessary to determine the remaining components of the reduced and alkylated methylated fibrinogen.

The two separated components from methylated fibrinogen were hydrolyzed with trypsin for various durations of time and the peptides released will be separated on a Dowex 50-X2 column utilizing ammonium formate buffers pH 3.2 to 5.0 at 50°C. We will attempt to isolate the methylated fibrinopeptides. Trypsin will hydrolyze the arginyl and lysyl bonds in the reduced and alkylated chains of methylated fibrinogen. Hopefully, the methylated fibrinopeptides, since they will be at the N-terminus of the chains, will be released quickly with short duration tryptic hydrolysis. To ensure that lysyl bonds, which the fibrinopeptides also contain, are not hydrolyzed by the trypsin, the ϵ-amino of the lysines was acetylated with maleic anhydride. The maleylated lysines now will not be susceptible to tryptic cleavage and only the arginyl bonds will be hydrolyzed. These peptide fragments (hopefully containing the intact methylated fibrinopeptides) from hydrolysis of methylated fibrinogen chains will be separated on Dowex 50 columns using various buffer systems.

It is believed a great deal can be learned from a study of these peptides and their behavior in physiological assay systems.

It is indeed interesting that, as we believe, the fibrinogen chains are kept apart by the high negative charge at the N-terminus of each chain. Then since the fibrinopeptides, which come from the ends of two of the fibrinogen chains possess high negative charge, and it is only after the action of thrombin which splits off these negatively charged peptides from fibrinogen, that the resulting fibrin molecules polymerize. It is therefore reasonable to assume that before the thrombin interaction, the fibrinogen chains are kept apart and prevented from polymerizing by the high repulsion imparted by the charge density in the vicinity of the fibrinopeptides. We therefore tested this theory by diminishing this negative charge at the end of the fibrinogen chain by methylating the carboxyls. What happened indeed was that the previously unpolymerized fibrinogen appeared to polymerize without the intervention of thrombin. Further investigation is in progress to further characterize the possible similarity between the fibrin clot and the polymerized fibrinogen. From the results to date, it appears that the mechanism of fibrinogen polymerization into the clot involves, as a first step, the removal of negative repulsion at the ends of two of the fibrinogen chains, thus enabling the resulting fibrin to polymerize into the clot formation. As the negative charge is removed through methylation, the fibrinogen obtains sufficient energy to polymerize independently of enzyme action.

Vasopressor effect of peptides released from fibrinogen by thrombin. The cat A peptide from fibrinogen, when injected intravenously into the rat, is unlike any of the previously described vasopressor fibrinopeptides. The cat A peptide did not produce any rhythmic vasoconstriction. Blood pressure levels remained constant or varied within experimental levels. It appears that by inspection of the structure of the cat peptide, a valine residue replaces the serine which is present in the canine peptide and therefore the cat peptide does not contain a hydroxylated amino acid in its N-terminus. This structure appears to be necessary for biological activity and all the fibrinopeptides tested to date that have been active either contained a serine or tyrosine residue near its N-terminus. This is quite interesting in that it implicates a particular amino acid as the active member of the "active site" of the vasoactive fibrinopeptides.

It is hoped to modify the cat peptide at its N-terminus to generate an hydroxyl group and then maybe this previously inactive peptide may become biologically active. This will enable us to further evaluate the possible role of fibrinopeptides as assisting in local hemostasis by vasoconstriction.

Modification of peptide released from fibrinogen by thrombin. The physiologically active canine A peptide from fibrinogen was dinitrophenylated to modify the N-terminus and the lone lysine residue in the molecule and to reduce the positive charges they generate in order to ascertain the mechanism of the effect or participation of these residues on the local hemostasis produced by vasoconstriction. The modified peptide was purified on Dowex and Sephadex ion exchange resins.

The canine peptide was also acetylated at the ϵ-amino of the lysine with maleic anhydride and thus change the charge that can be generated by the lysine functional group. The separation of the peptide was attempted on Dowex 1X2 in ammonium bicarbonate buffer pH 7.5 to 9.0 in 0.1 M sodium chloride. This modification of the canine A peptide will enable us to evaluate the participation of the positively charged lysine in the "active site" of the physiologically active material and to determine what influence it may have, if any, on the overall role of the peptides in hemostasis.

Significance of biomedical research and the program of the Institute: Our studies indicate a possible relationship of these peptides from fibrinogen to blood diseases and their effect on capillary pressures. The more we know about the structure relationships of these peptides to physiological activity, the easier will be the task of sorting out the little known mechanisms involved in furthering our understanding of certain blood related diseases.

Modification of the blood clotting proteins and peptides lead us to greater insight into the relationship between structure, physiological behavior and enzymatic activity. Ultimately we may better understand the overall mechanism of proteolytic enzyme action by applying the knowledge we obtain from the studies of the enzyme thrombin with its very narrow specificity. It is certainly easier to investigate the mechanism of action of a protease with a very narrow specificity than it would be to evaluate one with a wide range specificity.

Proposed course of project: Further modification and evaluation of structure and physiological role of peptides from fibrinogen will be investigated, as well as a continuation of the structural effects of the enzyme thrombin and the mechanism of its action on fibrinogen, specifically the possible action of thrombin on the modified fibrinogens as to bonds split, if any, and the influence of the modified residues on the enzyme action. Other modification methods will also be applied to fibrinogen in an effort to further evaluate its structural behavior.

Honors and Awards: None.

Publications: None.

ANNUAL REPORT SUMMARY
Laboratory of Molecular Biology

The work of the Laboratory of Molecular Biology is directed to an explanation of biological processes at the molecular level and to an understanding of the physical and chemical foundations of biological processes. Areas of investigation in the laboratory include study of the mechanisms of replication of DNA and of genetic recombination, studies of enzymes involved in recombination, characterization of mammalian and bacterial viruses by genetic analysis, studies of the molecular basis of viral properties, studies of the mechanism of protein synthesis, physical and chemical studies of the structure and properties of polynucleotides and nucleic acids, synthetic and spectroscopic investigation of polynucleotides, characterization of protein-DNA interaction in chromatin, investigation of protein structure by X-ray diffraction, and theoretical studies of nerve membrane characteristics. Major advances in several of these areas have been made by members of the Laboratory during the past year.

Transcription of chromatin

A method has been developed for measuring directly the number of RNA polymerase molecules that can bind to a given amount of template, under conditions which maintain chromatin in a completely soluble form. It is found that calf thymus DNA is capable of binding one *E. coli* polymerase molecule for each 1000 base pairs, while calf thymus chromatin binds one tenth as many molecules. Every polymerase molecule that binds is capable of initiating and propagating an RNA chain, though the rate of propagation in these low salt conditions is about 1/3 as fast on chromatin as on DNA. The conclusion is that chromatin is a restricted template for polymerase, and that the chromatin proteins serve to restrict activity by blocking initiation sites on the DNA, thus preventing enzyme binding.

These studies of template restriction have been extended to examination of *in vitro* production of a specific messenger RNA, that which is responsible for synthesis of globin in duck reticulocytes. To study the nature of template restriction in chromatin, purified duck reticulocyte chromatin was used as a template for the production of RNA *in vitro* with *E. coli* RNA polymerase. The purified RNA was then annealed against globin DNA. It was found that the transcript from reticulocyte chromatin contains globin RNA message to the extent of about one part in 10^4, comparable to its abundance in the large RNA isolated from reticulocyte nuclei *in vivo*. If duck liver chromatin or purified duck DNA were used as templates, no globin RNA was detected in *in vitro* transcripts. (Felsenfeld, Cedar, Axel, Stannard, Achter).

In related studies, the purification of polynucleotide kinase and polynucleotide ligase activities from rat liver nuclei has been undertaken, in order to test their interaction with the DNA in various regions and types of chromatin. (Zimmerman)

Studies on DNA replication

1) Replication of lambda phage DNA. Upon infection of *E. coli* bacteria,

λ phage DNA molecules form a covalently closed circular structure and replicate while keeping a circular form. To study the mechanisms of λ DNA replication, replicating DNA molecules in which newly synthesized progeny strands were labelled with ^{3}H-thymidine were isolated by the differential density labelling method previously developed in this group. Density gradient analysis of DNA after heat or alkaline denaturation showed that a certain fraction of newly synthesized DNA strands attached covalently to the parental DNA strands. The amount of the fraction of progeny strands which attached to the parental strands depended on the genetic constitutions of bacteria and phage used. When bacteria were $\underline{rec}^+$ and phage was $\underline{red}^+$ as much as 20 percent of the progeny strands attached to the parental strands. Both l and r strands of the progeny DNA were found in the attached progeny strands. When bacteria were recA and phage was red ($\beta^-\gamma^-$), the extent of attachment was several fold lower. These results suggest the participation of the functions primarily for genetic recombination in the process of attachment of progeny strands to parental ones. The results, however, do not tell whether the process is obligatory for initiation of DNA replication or secondary to it. The attachment of progeny strands to parental strands elongates the parental strands. Thus a certain fraction of infecting λ DNA molecules elongate even at the first cycle of replication. The fraction of molecules which multiply by elongation (rolling circle replication) increases later and replication by elongation becomes a predominant mode of replication in a late phase of phage growth. (Tomizawa)

2) Replication of extrachromosomal rDNA (DNA for ribosomal RNA) of Xenopus: Extrachromosomal rDNA replicates only at a certain time of the life cycle. The information for rDNA seems to exist originally in the chromosomes and replicated DNA forms circular structures of various sizes. These properties of rDNA mimic those of lysogenic phage DNA. Replication of rDNA in oocytes of excised ovaries was studied. Labelled DNA was extracted and analyzed by cesium chloride density gradient centrifugation. It was found that half-heavy rDNA appeared before the appearance of full-heavy rDNA during labelling with ^{3}H-bromodeoxyuridine. No measurable rDNA-rRNA hybrid molecules accumulated during incubation with ^{3}H-uridine. These data do not support the participation of reverse transcriptase (previously reported by others on the basis of unsatisfactory experiments) in the replication of rDNA. We conclude that rDNA replicates semi-conservatively. (Tomizawa, Sugino)

3) Genetic studies have been continued on DNA ligase, an enzyme required for DNA replication. By construction of suitable F' episomes which carry various alleles of the ligase genes, dominance relations in this region have been studied. Lig mutations are recessive to $\underline{lig}^+$, as expected for mutations in a structural gene, while lop mutations, which lead to overproduction of the enzyme, are cis-dominant, but trans-recessive, to $\underline{lop}^+$, in agreement with our previous idea that they affect a cis-acting control site (promoter or operator). (M. M. Gottesman, M. Gellert)

Functional Interaction of Bacterial Viruses with their Hosts

1) A bacterial mutant which restricts the growth of λ phage: Certain λ phage mutants kill all infected bacteria. Mutants of E. coli which survive

infection with one such phage (λc_{17}) were isolated and named *rol* (restriction of lambda). A λ mutant ($\lambda c_{17}\theta$) which could overcome the restriction imposed by the *rol* mutation was also isolated. Both the bacterial and phage mutations have been mapped; neither corresponds to any previously recognized function. The *rol* mutation does not block phage DNA replication at high temperature, but seems to protect the cell from the normally lethal effect of this replication; $\lambda\theta^+$ DNA also is not matured in these conditions. $\lambda\theta$ grows normally in the *rol* mutant at high temperature, but cannot help the growth of $\lambda\theta^+$. (Tomizawa, Sakakibara)

2) A host mutant which blocks the formation of phage heads: This *E. coli* mutant, called *mop*, prevents the growth of phages T4, λ, and $\phi 80$. In all cases the formation of phage heads is blocked.

The *mop* lesion seems to involve the cell membrane: cell permeability is altered, and one protein component of the membrane is missing. Thus a membrane-bound function is implicated in phage head morphogenesis.

Certain mutations in T4 gene 31 are able to overcome the effect of *mop*. Gene 31 is known to code for a function which catalyzes phage morphogenesis. These mutants have been exploited in setting up an *in vitro* system for T4 head morphogenesis. Complementation has been obtained between gene 23-defective extracts (gene 23 codes for the major head protein) and gene 31-defective extracts; however, the system only works if the gene 31 donor carries the mutation which can overcome *mop*. (T. Takano)

3) P1 is unique because it is a plasmid that has another life cycle as a vegetative virus. All other known plasmids, like F, R, or Colinogenic factors have only the cycle as a plasmid. In order to study the genetic and evolutionary relationships between these plasmids and P1-like bacteriophages, hybrids were made between P1 and R factors. These hybrids were as infective as the parental phage P1, and also carried resistance markers, originating from the R factor, to such drugs as kanamycin or ampicillin. These results suggest that P1 and other plasmids carry some genetically homologous regions and are evolutionarily related. The genetic and biophysical analysis of this homology is under investigation. (T. Takano)

4) Variants of bacteriophage P1 which are capable of specialized transduction of the bacterial *pro* genes have been isolated. Some of these P1s are deleted for various portions of the P1 genome, providing a means for unambiguous mapping of the P1 chromosome. Some others are not defective but give rise to defective phages upon induction. Errors in the P1 "head-full" mechanism for packaging DNA may be responsible for the origin of these non-defective phages. (Rosner)

5) A study has been made of λ "reverse" phages, a type originally described by Zissler. These are phages which, though originally deleted for recombination genes, have picked up a host DNA region again conferring recombination proficiency. It has now been shown by electron microscopic heteroduplex mapping that the substituting DNA fragment, about 10% of the λ genome in length, is identical in all λ reverse phages studied, despite their independent origins. (M. M. Gottesman, M. Gellert)

6) Development of λ in DNA ligase-defective hosts has been studied. In a λ-lysogenic host with a temperature-sensitive ligase, phage production is induced if the temperature is raised. This effect is not seen if the phage carries the ind$^-$ mutation in its repressor (ind$^-$ makes λ refractory to the normal induction by radiation or radiomimetic agents). Thus induction by a ligase defect is a repressor-mediated process, possibly acting *via* lesions in DNA similar to those resulting from irradiation. (Gottesman and Gellert)

Genetics and Structure of the Oncogenic Virus, SV40

Several lines of work have been pursued during the past year.

1) A number of temperature sensitive mutants of SV40 have been isolated. More will be collected before definitive characterization is undertaken.

2) Primary Chinese Hamster cells have been transformed by SV40 and are being cloned. The object is to determine whether the virus can integrate with only one type of chromosome or several different chromosomal types.

3) A complex of SV40 core proteins cross-linked to SV40 DNA has been characterized with the results suggesting that the core is randomly associated with the DNA rather than at a specific site.

4) Preliminary work is underway to characterize the tryptic peptides of the capsid proteins of SV40 virions. (Martin, Saral, Chou, Avila, McCann, and Brown).

Ion Transport Across the Nerve Membrane

1) In recent experimental work, H. Fishman was able to remove the non-specific 1/f K^+ current noise and hence analyze the remaining $1/f^2$ noise which is associated with the specific K^+ channels. His results are very similar to what we predicted in a previous paper (last year's report). We have therefore been encouraged to extend our earlier work on noise considerably. We have come to realize the use of noise measurements in analyzing biochemical and biophysical kinetic systems generally. Hence our new work applies to any steady-state or equilibrium first-order kinetic scheme. (Chen and Hill)

2) We have extended earlier work on cooperative models of membrane ion transport to an exact Monte Carlo analysis of a 10x10 Ising System (with periodic boundary conditions). In these models, it is assumed that the K^+ channels interact with each other. We show that, for practical purposes, 10x10 (periodic boundary conditions) is an "infinite" Ising system. The point of this work is to demonstrate that models of this type (still being pursued by G. Adam and others) cannot account for basic experimental facts. In particular, the K^+ current curves, in voltage clamp depolarizations, do not show the observed early induction behavior, or the observed superposition. (Chen and Hill)

Statistical Thermodynamics of Polynucleotide - Complementary Monomer Interactions.

1) The thermodynamics of small systems has been applied to the binding of ligands on dilute polymer molecules in solution, and especially to the binding of complementary monomers and oligomers on polynucleotides. Topics emphasized are: binding isotherms, optical melting curves, calorimetric melting and mixing studies, role of the activity of the ligand, quasi phase transitions, the polymer phase diagram, and interrelations between these subjects. The one-dimensional Ising model (linear and circular) was used to illustrate some of the thermodynamics. The main object here has been to provide experimentalists in the field with the proper thermodynamic framework with which to analyze and compare their results. (Hill)

2) A quite general theory of aggregation in solution has been worked out. The main application we have in mind is to the stacking of bases, nucleosides, etc., but this is just a special case. The theory applies to protein aggregation, etc. The new feature is to show how the (a) relatively strong forces leading to aggregation and (b) other forces of interaction between aggregates (usually ignored) can both be included in a single formulation, without approximation. At the least, "hard" interactions between aggregates should be included in many cases (e.g., base stacking). (Hill and Chen)

3) Exact expressions have been derived for the distribution function of the end-to-end vector of a random coil chain under the restriction that the chain makes a definite number of turns around a fixed point (2-dimensional case) or a fixed straight line (3-dimensional case). The ring-closure probability and the mean square end-to-end distance have also been calculated. Applications are to the entanglement of polymer chains, for example in circular double stranded DNA. (Saito and Chen)

4) In a parallel experimental study, an effort is being made to obtain a complete set of thermodynamic data on the system: adenosine-polyribouridylic acid-salt-water in order to rigorously account for the apparently discordant calorimetric and equilibrium measurements that have been reported for the binding of monomers to polynucleotides. (Ross, Scruggs)

Calorimetry of Biological Systems.

Two novel instruments have been developed in the past year:

1) A microcalorimeter designed to accept plastic petri dishes has been constructed for the purpose of studying cells grown in tissue culture. The thermokinetics of growth of 3T3-SV40 cells has been observed, demonstrating the feasibility of this approach.

2) A highly reliable differential scanning calorimeter for the study of thermally induced phenomena in solution has been constructed and is being tested. (P. Ross)

Studies on Polynucleotides.

1) It has been shown that polynucleotides can form helical structures even when the base-sugar bond is forced into the syn conformation (instead of the usual anti) by a bulky substituent on the 8-position of the purine. Furthermore, the complexes are as stable as comparable structures with the anti conformation. The molecules observed to date are a 1:1 monomer-polymer helix between 8-Br-GMP and poly BrC, and a 1:2 complex of poly 8-amino-adenylic acid and poly U. (Miles, Howard, Frazier)

2) The infrared absorption of the amide II vibration has been identified for the first time in a cis-amide, 1-methyluracil. The corresponding amide II band in poly U exhibits a large frequency increase (25 cm^{-1}) upon complex formation with poly A. This band is particularly valuable in permitting direct observation of hydrogen-bonding, and therefore in monitoring the helix-coil transition. (Miles, Lewis, Becker, Frazier)

3) A new two-stranded helix with non-Watson-Crick structure, poly 2,6-dimethyladenylic acid·poly U, has been made (in collaboration with Prof. Ikehara of Osaka University). Studies of this structure suggest that the intrinsic stability of non-Watson-Crick A·U helices is much lower than that of the corresponding Watson-Crick forms. (Miles, Hattori)

4) Using light scattering, the radius of gyration of short poly A chains at low temperature has been measured. The radius of gyration reaches a limiting value as the temperature decreases. The dependence of the limiting value of the radius of gyration on chain length has been measured using well-defined molecular weight fractions of poly A. It is found that the radius of gyration varies as the first power of the molecular weight, as expected for a rigid rod. The data are consistent with a rod of length 3.2A per nucleotide, quite reasonable for a polynucleotide. (Felsenfeld, Achter, Stannard).

5) Fibers of poly G giving interpretable x-ray diffraction patterns have been prepared. There are relatively few data in the pattern because of the high symmetry of the structure. Based on several lines of evidence, it is found that the pattern best fits a four-stranded structure. Because of the great similarity between the patterns given by poly G and poly I, it seems quite likely that poly I also forms a similar four-stranded structure rather than the three-stranded structure proposed by Rich. (Zimmerman)

6) The precipitation of polynucleotides at acid pH has been studied. Polynucleotides precipitate at well defined pH values. These pH values are dependent upon both the base composition and the ionic strength of the medium. At low ionic strength, all the polymeric components in solution tend to precipitate together. The pH value at which such coprecipitation occurs can be predicted based upon a simple model which required charge neutralization between the bases and phosphate residues for insolubility, i. e., precipitation occurs at the net isoelectric point of the solution.

At higher ionic strengths, there were instances of fractional precipitation. Also at higher ionic strenghts, several homopolymers (poly A, poly G, and to a lesser extent, poly U) had a second zone of insolubility near neutral pH values. (Zimmerman)

X-ray Crystallography of Proteins.

1) The atomic coordinates of tosyl-γ-chymotrypsin as obtained from a 2.7 Å resolution electron density map are being refined. It is hoped that it will be possible to transfer the results to the native enzyme and extend the resolution to 2.0 Å. (Davies, Cohen, Subramanian)

2) More heavy atom derivatives of the acid protease from Rhizopus chinensis are being sought in an effort to improve the quality of the presently uninterpretable 3.1 Å resolution electron density map. (Davies, Swan)

3) A search for crystalline Fab antibody fragments with antibody activity has continued; a second type, J539, suitable for high resolution X-ray analysis, has been obtained.

4) A 4.5 Å map has been calculated for the Fab antibody fragment McPC 603, and the overall shape determined. The antigen binding site has been located, and lies at one end of the Fab fragment, in the cleft formed at the junction of the H and L chains. Data collection for a 3.1 A map is well underway.

The 4.5 Å map of McPC 603 has been compared with the 6 Å map of the hyman cryoglobulin Dob. (Davies, Segal, Padlan, Silverton)

Protein Synthesis in Mammals.

Investigations have continued on the chemical and enzymatic properties of EF-1 and EF-2, the two soluble protein factors required for polypeptide chain-elongation in eukaryotic cells. Multiple forms of EF-1 were resolved by several techniques, but no functional differences in the various forms were found.

A study of the interaction of guanosine nucleotides with EF-2 was carried out by equilibrium dialysis. The k_d for GDP is 4×10^{-7} and that for GTP is 2×10^{-6}. There appears to be one site for each of the two nucleotides per molecule of EF-2. The complex relationship between enzyme-bound GDP and GTP points to a possible control mechanism.

A tryptic peptide containing ^{14}C-ADP-ribose has been isolated from ^{14}C-ADP-ribose-EF-2 and its amino acid composition has been determined. Isolation of this peptide is a step toward elucidating the site of covalent attachment of ADP-ribose to ADP ribosyl-EF-2 and of determining the amino acid sequence around this active site of the enzyme. (Maxwell, Collins, Henriksen, Tudor)

Base Sequence Analysis of R17 phage RNA.

Methods have been developed for sequencing very large RNA molecules. The critical step is controlled enzymatic cleavage to yield defined large fragments. With the R17 RNA which is being used as a model, this step was carried out with ribonuclease T1. Two pairs of fragments which can be considered to have been cleaved from the whole molecule by a single action of RNase T1 were found. The cleavages were produced at points 36 and 47 percent from the 5' end of R17 RNA. (Fuke)

Serial No.: NIAMDD-LMB-1
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Crystal structure investigation of antigen antibody interaction

Previous Serial Number: NIAMDD-LMB-11

Principal Investigators: David R. Davies, D. M. Segal, E. A. Padlan, and E. W. Silverton

Cooperating Units: Dr. Michael Potter, NCI; Dr. Stuart Rudikoff, NCI; and Dr. Thomas Spande, NIAMDD

Man Years:

Total: 3-1/4
Professional: 3-1/4
Others: 0

Project Description:

Objectives: A comparative investigation of the crystal structures of the Fab fragment of immunoglobulin molecules that bind strongly to several different antigens. In this way, it is hoped to map out the binding site of antibodies and to illustrate the mechanism of antigen-antibody interaction.

Methods Employed: Preparation and analysis of pure materials. Crystallization and x-ray diffraction analysis with the heavy atom isomorphous replacement method.

Major Findings: 1) During the past two years some 16 Fab fragments have been received in reasonably pure form from Drs. Potter and Rudikoff. These are the Fab fragments from mouse myeloma proteins where the intact immunoglobulin is known to bind very strongly to small molecules such as phosphoryl choline. To date crystals have been obtained from seven of these immunoglobulin fragments.

a) MOPC 167 space group P622, cell dimensions a=114, b=114, c=146 Å.
b) McPC 603 space group P63, a=161, b=161, c=60.3 Å.
c) SAPC 10 space group = P6$_n$22, cell dimensions a=139, b=139, c=97.5 Å.
d) MOPC 460 crystals not yet suitable for x-ray analysis.
e) J539 space group P2,2,2, cell dimensions a=55, b=75, c=131 Å.
f) XRPC 24 do not diffract.
g) MOPC 315 FV do not diffract.

Crystals of McPC Fab' diffract to at least 3 Å and have been subjected to x-ray analysis. Suitable heavy atom derivatives were obtained with thulium chloride and with $K_2Pt(CNS)_6$. An electron density map at 4·5 A resolution showed clearly the outline of the molecule. The overall appearance is similar to that observed by Poljak et al. in a human Fab', the molecule consisting of two globular regions, each of which is in turn composed of two domains.

When the crystals are soaked in a solution containing a heavy atom-containing analogue of phosphoryl choline, acetoxymerurithienyl phosphoryl choline (AMTEPC), they yield a diffraction pattern with significant intensity changes. Difference electron density maps show that the AMTEPC binds in two sites, one of which is identical with the platinum binding site. Displacement studies with phosphoryl choline showed that the AMTEPC could be displaced from the second site but not from the first. We conclude that the former is the true antigen binding site. Support for this conclusion comes from the location of the site, at the end of the molecule, in the cleft between two domains.

Comparisons between the electron density map of the immunoglobulin Dob and the constant domains of the McPC603 Fab show that in the F_c region the domains have the same overall appearance and relative orientation. In addition to McPC603, crystals suitable for x-ray analysis have been grown from arabino-galactan binding protein, J539.

Significance to Bio-medical Research and the Program of the Institutes: An understanding of the molecular basis of interaction between antibody and antigen is fundamental to our understanding of the immune response.

Proposed Course: We plan to complete the structure determination of McPC603 Fab and commence the analysis of J539.

Publications:

Rudikoff, S., Potter, M., Segal, D. M., Padlan, E. A., and Davies, D. R.: Crystals of Phosphorylcholine-binding Fab-Fragments from Mouse Myeloma Proteins: Preparation and X-ray Analysis. Proc. Nat. Acad. Sci. USA 69: 3689-3692, 1972.

Serial No.: NIAMDD-LMB-2
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: X-ray diffraction investigation of proteolytic enzymes

Previous Serial Number: NIAMDD-LMB-12

Principal Investigators: David R. Davies, Gerson H. Cohen, Ian D.A. Swan, and E. Subramanian

Man Years:

Total: 1-3/4
Professional: 1-3/4
Others: 0

Project Description:

Objective: The determination of the 3-dimensional structure of proetolytic enzymes, in particular, the structure of gamma chymotrypsin and of the acid protease from Rhizopus chinensis.

Methods Employed: 1. The refinement of the coordinates of tosyl γ-chymostypsin at 2.7 A resolution has been continued. The refined coordinates are being used to compute structure factors for the native enzyme whose resolution will then be extended to 2 A and refined further. The refinement is being done with a) a physical model, b) a computer display, and c) conventional crystallographic computations.

2. The 3-dimensional electron density map for the acid protease from Rhizopus chinensis at 3.1 A resolution was calculated. It turned out to be uninterpretable without the amino acid sequence, although the general periodicities apparent in the map were very encouraging. A new search for new derivatives which will provide an interpretable map has been commenced.

Significance to Bio-medical Research and the Program of the Institute: The work on the mode of binding of specific inhibitors to chymotrypsin has turned out to have fairly universal application and has been used to show how trypsin and trypsin inhibitor interact. It has also thrown light on the evolutionary mechanism for the production of enzymes. An understanding of the crystal structure of the acid protease and of its mechanism of action will be of fairly universal interest with regards to the molecular mechanism of action and control of this important class of enzymes.

Proposed Course of Project: When the electron density map of the acid protease from Rhizopus chinensis becomes available, an attempt will be made to interpret this in molecular terms. A search will be made for suitable inhibitors to reveal the location and stereo chemistry of the active site. Experiments will be designed to reveal the mechanism of action of the enzyme.

Serial No. NIAMDD-LMB-3
1. Laboratory of Molecular Biology
2. Section on Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Enzymatic Joining of DNA Strands and its Role in Genetic Recombination

Previous Serial Number: NIAMDD-LMB-1

Principal Investigator: Martin Gellert

Other Investigator: Michael M. Gottesman
Kiyoshi Mizuuchi
Minnie L. Hicks

Man Years:		
	Total:	3 1/3
	Professional:	2 2/3
	Other:	2/3

Project Description:

Objectives: To understand the physiological role of DNA ligases; to study the biochemical basis of genetic recombination.

Methods Employed: Ultracentrifugation, standard enzymological and microbiological techniques, and electron microscopy.

Major Findings: 1) DNA ligase mutants of *E. coli*. (M. Gellert, M. M. Gottesman, M. L. Hicks).

a) DNA replication: We had previously shown that *lig* mutants accumulate short DNA chains in pulse-labeling experiments, to a much greater extent than normal strains. The nature of this DNA has now been studied. By a variety of tests, it is found to be a predominantly single-stranded and thus dissociated from the parental template strand. The further processing of this DNA is being studied.

b) λ phage induction: Phage production is induced from λ lysogens in a host with a temperature-sensitive ligase if the temperature is raised. This effect is not seen if the phage carries the ind^- mutation in its repressor (ind^- makes the phage refractory to the normal induction by radiation or radiomimetic agents). Thus induction by a ligase defect is a repressor-mediated effect, possibly, acting *via* lesions in DNA similar to those produced by radiation.

2) Studies on "reverse mutants of λ. (M. M. Gottesman, M. Gellert).

Starting from λ phages deleted for their recombination genes, reverse mutants (originally described by J. Zissler) have reacquired recombination proficiency by some interaction with the host chromosome. We have isolated two such phages from different λlysogens (with λ inserted in ptsI and trpC respectively). By electron microscopic heteroduplex mapping involving these two phages and one of Zissler's isolates, we have shown that all three phages carry the same substitution of host DNA, with identical left and right end points.

Enzyme assays show that these phages code for a nuclease activity distinct from λ exonuclease and possibly identifiable with exonuclease VIII, a host enzyme newly discovered by A.J. Clark and others.

Serial No. NIAMDD-LMB-4
1. Laboratory of Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Protein Synthesis in Mammals: Mechanisms and Metabolic Controls

Previous Serial Number: NIAMDD-LMB-2

Principal Investigator: Elizabeth S. Maxwell

Other Investigators: James F. Collins
Elizabeth Tudor
Ole Henriksen

Man Years:
Total: 3 1/2
Professional: 3 1/2

Project Description:

Objectives: Our overall objective is to gain an understanding of the mechanisms and metabolic controls of protein synthesis in mammals. We hope to obtain a partial understanding of these processes through detailed knowledge of the soluble enzymes involved in polypeptide chain initiation, elongation and termination.

Methods Employed: All of the methods employed are either standard procedures or minor modifications thereof.

Major Findings: Investigations of the chemical and enzymatic properties of EF-1 and EF-2, the two soluble protein factors required for polypeptide chain-elongation in eukaryotic cells have continued.

Multiple forms of EF-1 were resolved by several techniques, but no functional differences in the various forms were found.

A study of the interaction of guanosine nucleotides with EF-2 was carried out by equilibrium dialysis. The Kd for GDP is 4×10^{-7} and that for GTP is 2×10^{-6}. There appears to be one site for each of the two nucleotides per molecule of EF-2. The complex relationship between enzyme-bound GDP and GTP points to a possible control mechanism.

A tryptic peptide containing ^{14}C-ADPribose has been isolated from ^{14}C-ADPribosyl-EF-2 and its amino acid compositions has been determined. Isolation of this peptide is a step toward our goal of illucidating the site of covalent attachment of ADPribose to ADPribosyl-EF-2 and of determining the amino acid sequence around this active site of the enzyme.

Significance to NIAMDD Research: The enzymatic mechanisms in protein synthesis in mammals and the metabolic control of these processes is of major interest to biology and biochemistry. Our emphasis so far is on enzymatic mechanisms. Hopefully, understanding of control will follow.

Proposed Course of Project: We intend to pursue our investigation of the various steps involved in the overall process of protein biosynthesis in mammalian cells. Our aim is to have all the components necessary for the process purified in order to be able to synthesize specific proteins in vitro and to study the control of their formation.

Further chemical characterization of EF-2 and its ADPR derivative will continue until we have determined the site of attachment of ADPribose and the sequence of amino acids adjacent to it.

Publications:

Collins, James, F., Moon, Hong, Mo, and Maxwell, Elizabeth, S.: Multiple Forms and Some Properties of Aminoacyltransferase I (Elongation Factor 1) from Rat Liver. Biochemistry 11: 4187-4194, 1972

Robinson, Elizabeth, A., and Maxwell, Elizabeth, S.: Chemical Properties of Elongation Factor 2, Amino Acid Composition, NH_2-Terminal Residue, and Sulfhydryl Reactivity. The Journal of Biological Chemistry. 247: 7023-7028, 1972.

Schneider, Jerry, A and Maxwell, Elizabeth, S.: Peptidylpuromycin Formation on Transpeptidation and Translocation. Biochemistry: 12, 475 - 481. 1973.

Serial No. NIAMDD-LMB-5
1. Laboratory of Molecular Biology
2. Section on Organic Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Chemical and Structural Investigations of Nucleic Acids and Related Substances

Previous Serial Number: NIAMD-LMB-13

Principal Investigator: H. Todd Miles

Other Investigators: Dr. F. B. Howard, Dr. M. Hattori, and Mr. J. Frazier

Cooperating Units: Dr. E. D. Becker (LCP, NIAMDD) and Dr. M. Ikehara (University of Osaka)

Man Years:

Total:	4-1/2
Professional:	3-1/2
Other:	1

Project Description:

Objectives: Basic chemical, biochemical, and structural studies of nucleic acids and related substances.

Methods: Infrared spectroscopy, nuclear magnetic resonance spectroscopy, chemical synthesis and structure elucidation, optical rotation, ultraviolet spectroscopy, use of enzymes for synthesis and structure study.

Major Findings: Purine and pyrimidine nucleosides are known to exist in conformationally isomeric forms, which can be described by the angle of rotation about the glycosidic bond joining the sugar to the base. Extensive study in a number of laboratories has shown that anti conformations are strongly favored, but that both syn and anti structures are capable of existence. Though there has been no evidence on the effect of syn conformations on the structure of macromolecules, the only explicit statements in the literature suggest that the existence of nucleotide residues in a syn conformation would prevent helix formation. We have been able to demonstrate, however, that helix formation can in fact occur when the purine nucleotide residues are in a syn configuration. The stabilities of the helices, moreover, are similar to those of comparable complexes in which all the residues have an anti configuration.

We have employed model systems in which the nucleotides are sterically forced into a syn configuration by bulky residues in the 8-position of the purine nucleotides. The molecules observed to date are a 1:1 monomer-polymer helix which we have demonstrated between 8-Br-GMP and poly BrC and a 1:2 complex formed between poly 8-aminoadenylic acid and poly U (Howard, Frazier, and Miles).

The amide II band is the second vibrational transition of the CONH group in secondary amides (the first band is the familiar carbonyl stretching vibration). This band has been extensively studied in secondary trans amides but has generally been reported to be absent from the spectra of cis amides. We have now identified the amide II band at 1416 cm^{-1} in the cis amide 1-methyluracil. Infrared and Raman spectra of an extensive series of isotopic derivatives provide conclusive support of the assignment and show that the N-H in-plane bending vibration is extensively coupled to both carbonyl vibrations as well as to motions of the N-1 and C-6 atoms. We have identified the corresponding amide II band in poly U and in the complex formed by poly A and poly U. The band in this helical complex exhibits a large frequency increase (25 cm^{-1}) over the unperturbed value (1422 cm^{-1}) in the random coil. N-H stretching vibrations of the hydrogen-bonded protons of nucleic acids cannot be observed in aqueous solution because of much stronger absorption by the solvent. Identification of the amide II band is therefore particularly valuable in permitting direct observation of the hydrogen-bonded proton in aqueous solution. From the frequency and intensity changes of the amide II band it is possible to tell whether the proton is hydrogen bonded and to use the band to monitor the helix-coil transition (Miles, Lewis, Becker, and Frazier).

We have shown that two-stranded non-Watson-Crick A·U helices are capable of existence, though the stabilities of the observed complexes (poly $2NMe_2A$·poly U and poly $2NMe_2A$·poly BrU) are much lower than those of the corresponding Watson-Crick helices. It was not clear, however, whether the lower stability was intrinsic or was due to a non-specific destabiling effect of the NMe_2 group (Ishikawa, Howard, Frazier, and Miles).

We have now investigated, in collaboration with Prof. Ikehara of Osaka University and Dr. Hattori of LMB, the base-pairing properties of poly 2,6-dimethyladenylic acid. This polynucleotide also forms a non-Watson-Crick helix of low stability with poly U. In contrast to the Watson-Crick helix having a 6-NMe group introduced into poly A (Ikeda, Frazier and Miles, 1970), however, the 6-NMe group is not significantly destabilizing in this non-Watson-Crick helix. Though our investigation of the subject is not complete, the new results suggest that the intrinsic stability of the non-Watson-Crick A·U helices is indeed much lower than that of the corresponding Watson-Crick helices. As we have pointed out in the paper of Ishikawa et al, this conclusion is not required by the fact that only a Watson-Crick helix is observed with poly A and poly U. The strongly cooperative nature of the helix-coil transition would assure exclusive formation of a single species, even with only a small difference of stability.

We have found that 5'-guanylic acid forms in neutral solution a regular, ordered structure, presumably helical. This structure is different from that previously reported at pH 5 and is not associated with gel formation. The transition is more cooperative than that of the pH 5 helix but has a lower Tm. In the neutral or slightly alkaline solutions investigated we are probably dealing with isotropic solutions of helices of quite low molecular weight, even at relatively high concentrations of nucleotide (Miles and Frazier).

Publications:

Ishikawa, F., Frazier, J., Howard, F. B., and Miles, H. T.: Polyadenylate·polyuridylate helices with non-Watson-Crick hydrogen bonding. J. Mol. Biol. 70: 475-490, 1972.

Miles, H. T., and Frazier, J.: Formation of a new 5'-guanylic acid helix in neutral solution. Biochem. Biophys. Res. Comm. 49: 199-204 (1972).

Howard, F. B., Frazier, J., and Miles, H. T.: Formation of polynucleotide helices having purine nucleotide residues in a syn configuration. J. Biol. Chem. 247: 6733-6735, 1972.

Miles, H. T., Lewis, T. P., Becker, E. D., and Frazier, J.: Identification of the amide II band in the cis-amide I-methyluracil, in poly(U), and in poly(A)·poly(U). J. Biol. Chem. 248: 1115-1117, 1973.

Serial No. NIAMD-LMB-6
1. Laboratory of Molecular Biology
2. Section on Molecular Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Replication, Recombination and Repair of Microbial DNA

Previous Serial Number: NIAMD-LMB-10

Principal Investigator: J. Tomizawa (11/12)

Other Investigators: Y. Sakakibara (12/12)
Y. Sugino (12/12)
A. C. Satterthwait (2/12)

Man Years:

Total:	3
Professional:	3
Other:	0

Project Description:

Objectives: Molecular biological understanding of the mechanisms of replication, recombination and repair of DNA is aimed.

Methods Employed: The mechanisms are studied mainly with *E. coli* and phages. Various genetic tricks are used extensively. Density gradient centrifugation (CsCl and sucrose), differential labelling of DNA with radioisotopes and heavy isotopes were used for isolation and analysis of DNA. Structures of DNA were visualized by the electron-microscopic method.

Major Findings:

1) Replication of lambda phage DNA. Upon infection to *E. coli* bacteria, λ phage DNA molecules form a covalently closed circular structure and replicate while keeping a circular form. To study the mechanisms of λ DNA replication, replicating DNA molecules in which newly synthesized progeny strands were labelled with ^{3}H-thymidine were isolated by the differential density labelling method we developed. Density gradient analysis of DNA after heat or alkaline denaturation showed that a certain fraction of newly synthesized DNA strands attached covalently to the parental DNA strands. The amount of the fraction of progeny strands which attached to the parental strands depended on the genetic constitutions of bacteria and phage used. When bacteria were rec$^+$ and phage was red$^+$ as much as 20 percent of the progeny strands attached to the parental strands. Both *l* and *r*

strands of the progeny DNA were found in the attached progeny strands. When bacteria were recA and phage was red ($\beta^-\gamma^-$), the extent of attachment was several fold lower. These results suggest the participation of the functions primarily for genetic recombination in the process of attachment of progeny strands to parental ones. The results, however, do not tell whether the process is obligatory for initiation of DNA replication or secondary to it. The attachment of progeny strands to parental strands elongates the parental strands. Thus a certain fraction of infecting λ DNA molecules elongate even at the first cycle of replication. The fraction of molecules which multiply by elongation (rolling circle replication) increases later and replication by elongation becomes a predominant mode of replication in a late phase of phage growth.

2) A bacterial mutant which restricts the growth of lambda phage. λ phage carrying a promoter mutation $\underline{c}_{17}$ replicates constitutively and consequently the infected bacteria are killed. When the bacteria are infected with $\lambda\underline{c}_{17}$ carrying susO or susP mutation which is defective in DNA replication, the cells are not killed. Bacterial mutants which survive infection with $\lambda\underline{c}_{17}$ were isolated. One of the mutants supported the growth of wild type λ phage at low temperature but not at high temperature. The bacterial mutation (rol : restriction of lambda) was mapped between lysA (55 min.) and metC (59 min.). A λ mutant which could overcome the restriction by the bacterial mutation was isolated. The mutation (θ) was mapped at 39.5% scale of molecular distance of lambda DNA and between gene J and b_{519}. DNA synthesis of $\lambda\underline{c}_{17}$ in the rol mutant at the non-permissive temperature was not significantly different from that of $\lambda\underline{c}_{17}\theta$. The rol mutant was not killed by the infection with λ at high temperature although DNA replication of λ was not blocked. Therefore, the rol mutation seems to protect the infected bacteria from killing by the λ DNA replication. λθ could grow normally in the rol mutant at high temperature but not help the growth of the coinfected $\lambda\theta^+$. In the rol mutant, $\lambda\theta^+$ DNA synthesized at high temperature does not mature because of some defect in DNA itself or of lack of some cis-acting factor required for the particle formation.

3) Replication of extrachromosomal rDNA (DNA for ribosomal RNA) of Xenopus. Although the material used is not a microorganism, apparent similarity of mode of replication of rDNA of Xenopus and microbial DNA as described below and application of the techniques developed for microbial genetics may allow us to report this result as a part of this project report.

Extrachromosomal rDNA replicates only at a certain time of the life cycle. The information for rDNA seems to exist originally in the chromosomes and replicated DNA forms circular structures of various sizes. These properties of rDNA mimic those of lysogenic phage DNA.

In spite of this apparent similarity, practically nothing is known about the mechanism of rDNA replication. Based on unsatisfactory experiments, the participation of reverse transcriptase has been claimed.

We studied replication of rDNA in oocytes of excised ovaries. The DNA was labelled with ^{3}H-thymidine, ^{3}H-bromodeoxyuridine, ^{3}H-uridine or $H_3{}^{32}PO_4$. DNA was then extracted and analyzed by cesium chloride density gradient centrifugation. We found that half-heavy rDNA appeared before the appearance of full-heavy rDNA during labelling with ^{3}H-bromodeoxyuridine. No measurable rDNA-rRNA hybrid molecules accumulated during incubation with ^{3}H-uridine. These data do not support the participation of reverse transcriptase in the replication of rDNA. We conclude that rDNA replicates semi-conservatively.

Significance to Bio-medical Research and the Program of the Institute: Understanding of the mechanisms of replication, recombination and repair has prime importance in genetics. Extensive investigation can be made with the system with which we are working. Understanding of these processes will have significance for bio-medical research and the program of the Institute.

Proposed Course of Project: Studies on the mechanisms of initiation of replication of DNA and those of genetic recombination will be continued. *In vitro* systems for replication and recombination of DNA will be developed.

Serial No. NIAMDD-LMB- 7
1. Laboratory of Molecular Biology
2. Section on Molecular Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Base Sequence Analysis of R17 Phage RNA

Previous Serial Number: NIAMD-LMB-9

Principal Investigator: M. Fuke (12/12)

Other Investigator: J. Tomizawa (1/12)

Man Years:

Total:	13/12
Professional:	13/12
Other:	0

Project Description:

Objectives: Determination of the complete base sequence of R17 phage RNA is aimed through the development of a method to obtain unique collections of fragments of the RNA.

Methods Employed: Limited and controlled digestion of R17 RNA by ribonuclease T1 and electrophoretic separation of the RNA fragments by acrylamide gel electrophoresis.

Major Findings: Large fragments of R17 RNA were obtained using limited digestion by ribonuclease T1. Two pairs of fragments which can be considered to have been cleaved from the whole molecules by a single action of ribonuclease T1 were found. The cleavages were produced at points 36 and 47 percent from the 5' end of R17 RNA.

Significance to Bio-medical Research and the Program of the Institute: Development of a method which is widely applicable for specific fragmentation of nucleic acids has fundamental importance for nucleic acid chemistry and biology. Sequence analysis of R17 RNA will allow us to explain various functions of the phage based on RNA structure. This research will increase fundamental knowledge of bio-medical knowledge and help to understand RNA oncogenic viruses which carry small single stranded RNA as R17 phage carries.

Proposed Course of Project: This research will be continued. Further fractionation will be performed and the ends newly formed by ribonuclease T1 will be analyzed.

Serial No. NIAMDD-LMB - 8
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Structure of Single Strand Polynucleotides in Solution

Previous Serial Number: NIAMDD-LMB-17

Principal Investigator: Gary Felsenfeld

Other Investigators: E. Achter
B. J. Stannard

Man Years:

Total: 1-1/4
Professional: 1-1/4
Others: 0

Project Description:

Objectives: To elucidate the structure of single strand polyribonucleotides in solution.

Methods Employed: Theoretical analysis, light scattering, viscometry, spectrophotometry, sedimentation velocity, sedimentation equilibrium.

Major Findings: We have continued our study of polyadenylic acid (poly A) conformation in solution. At sufficiently low temperature (0 to 10°C), the bases are almost completely in the "stacked" configuration, as judged by optical properties. Using light scattering, we have measured the radius of gyration of short poly A chains in this low temperature region. We find that the radius of gyration reaches a limiting value as the temperature decreases. The dependence of the limiting value of the radius of gyration on chain length has been measured, using well defined molecular weight fractions of poly A. We find that the radius of gyration varies as the first power of the molecular weight, as expected for a rigid rod. The data are consistent with a rod of length 3.2Å per nucleotide, quite reasonable for a polynucleotide.

We have also begun detailed studies of the low temperature behavior of polydeoxyadenylic acid, in order to compare the analogous deoxyribose polymer with the ribose polymer.

Significance to Bio-medical Research and the Program of the Institute: The results for poly A at low temperature represent the first information about the dimensions of single strand ordered polynucleotide structures, which it has not been possible to study by X-ray diffraction. The results also provide further information about the temperature-dependent behavior of this prototypical single strand polynucleotide. As a result of our studies, the conformation of poly A at all temperatures is now known, and the temperature dependent behavior can be accounted for in theoretical terms as well. The conformation of such molecules is a major determinant in their chemical behavior, and this behavior plays a dominant role in the reactivity of naturally occurring RNA, such as messenger RNA, viral RNA, ribosomal and transfer RNA, under natural conditions in the living cell.

Proposed Course of Project: We plan to continue our studies, with emphasis upon the deoxyribose polymers, and upon the role of the solvent in structural stabilization.

Serial No. NIAMDD-LMB-9
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Chromatin Structure and Function

Previous Serial Number: 18

Principal Investigator: Gary Felsenfeld

Other Investigators: Howard Cedar, Richard Axel, Bethel Stannard and Eugene Achter

Man Years:

Total: 3-3/4
Professional: 3-3/4
Others: 0

Project Description:

Objectives: The object of this project is to determine the structure of chromatin and the relationship of this structure to its biological role in cell differentiation.

Methods Employed: Spectrophotometry, analytical ultracentrifugation, dialysis equilibrium, and other standard techniques are used to study the binding of charged molecules to DNA, and the composition of the regions of DNA to which these molecules are bound. Standard chromatographic and electrophoretic techniques are used in purification and identification of histones. RNA polymerase is purified by classical enzymological techniques and its activity examined by membrane filtration, sucrose gradient sedimentation, or radioisotope incorporation. DNA-RNA hybridization methods are used to detect presence of specific polynucleotide sequences.

Major Findings: We have continued our studies of the chemical reactivity of chromatin, and its relation to chromatin structure. It has long been thought that the proteins of chromatin serve to restrict its activity as a template for transcription by DNA dependent RNA polymerase, in this way giving rise to cellular differentiation. We have therefore examined the binding of RNA polymerase from E. coli to chromatin from calf thymus and duck reticulocyte. Previous methods for studying this interation suffer from two major difficulties: The chromatin is insoluble under the usual reaction conditions and the reaction conditions are not chosen in such a way that the amount of reaction is proportional to the amount of template DNA available. We have found a way to study the polymerase-chromatin interaction under conditions which maintain the chromatin in a completely soluble form. In addition, we have developed a method which permits us to measure directly the number of polymerase molecules that can bind to a given amount of template. We find that calf thymus DNA is capable of binding one E. Coli polymerase molecule for each 1000 base pairs, while calf thymus chromatin binds one tenth as many molecules. Every polymerase molecule that binds is capable of initiating and propagating an RNA chain, though the rate of propagation in our low salt conditions is about 1/3 as fast on chromatin as on DNA. We conclude that chromatin is a restricted template for polymerase, and that the chromatin proteins serve to restrict activity by blocking initiation sites on the DNA, thus preventing enzyme binding.

We have extended these studies of template restriction to examination of in vitro production of a specific messenger RNA, that which is responsible for synthesis of globin in duck reticulocytes. Using methods developed by others, we have isolated globin message RNA from reticulocytes and used RNA-dependent DNA polymerase to produce from the globin RNA a radioactive DNA chain with a base sequence complementary to that of the globin RNA. This globin DNA can be used in DNA-RNA hybridization experiments as a probe for the presence of globin messenger RNA in unknown RNA samples. To study the nature of template restriction in chromatin, we have purified duck reticulocyte chromatin and used it as a template for the production of RNA in vitro with E. coli RNA polymerase. The purified RNA is then annealed against globin DNA. We find that the transcript from reticulocyte chromatin contains globin RNA message to the extent of about one part in 10^4, comparable to its abundance in the large RNA isolated from reticulocyte nuclei in vivo. If duck liver chromatin or purified duck DNA are used as templates, no globin RNA is detected in in vitro transcripts.

Significance to Bio-medical Research and the Program of the Institute: These results provide the first conclusive evidence for transcriptional control of gene expression in eukaryotes. They also are the first conclusive proof that chromatin restricts transcription in a tissue-specific manner, and that the restriction is similar to that observed in vivo. Thus, the chemical basis of differentiation is inherent in this purified complex of protein and DNA, and can be studied outside the nucleus. The results are important to all studies of differentiation, and the growth of normal and abnormal eukaryotic cells. The method we have developed should make possible detection of the specific proteins involved in the differentiation process.

Proposed Course of Project: We are carrying out investigations of the relationship between the presence of various chromatin protein components and the restriction of template activity, using globin message production as a test of biological activity. We hope to find the proteins responsible for the activation of globin gene transcription and to shed light on the chemical basis of differentiation.

Publications:

Clark, R. J. and Felsenfeld, G.: Association of Arginine-rich Histones with G·C-rich Regions of DNA in Chromatin. Nature New Biology 240: 226-229, 1972.

Cedar, H. and Felsenfeld, G.: Transcription of Chromatin In Vitro. Journal of Molecular Biology, in press.

Axel, R., Cedar, H. and Felsenfeld, G.: The Synthesis of Globin RNA from Duck Reticulocyte Chromatin In Vitro. Proceedings of the National Academy of Sciences, in press.

Serial No. NIAMDD-LMB-10
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Calorimeter for the Study of Cells grown in Tissue Culture

Previous Serial Number: (none)

Principal Investigator: Philip D. Ross

Other Investigator: (none)

Man Years

Total	1/3
Professional:	1/3
Other:	0

Project Description:

Objectives: To observe and interpret the thermokinetic behavior of cells grown in tissue culture.

Methods Employed: A differential microcalorimeter designed to accept 35mm Falcon plastic petri dishes has been constructed. A well thermostatted environment controlled to ± 40 microdegrees confers long term thermal stability on the instrument. The stability of the instrument makes observation possible over a period of several days, thus very small energy changes associated with the growth of cells in tissue culture may be detected.

Major Findings: The calorimeter described above has been constructed and growth of 3T3-SV40 cells has been observed demonstrating the feasibility of this approach.

Significance to Bio-Medical Research and the Program of the Institute: The methods developed in this work may now be applied to a variety of interesting and significant experimental situations. It is planned to investigate the growth patterns of different cell lines and to study the effects of various agents upon the heat production of tissue culture grown cells.

Proposed Course of Project: This work is being continued.

Serial Number: NIAMDD:LMB-11
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Differential Scanning Calorimeter

Previous Serial Number: (none)

Principal Investigator: Philip D. Ross

Other Investigator: Robert N. Goldberg

Man Years:

Total: 2/3
Professional: 2/3
Other: 0

Project Description:

Objectives: To study thermally induced phenomena in solution.

Methods Employed: A scanning calorimeter utilizing the principles of heat conduction calorimetry has been constructed. Its temperature may be raised at rates up to 16°K per hour by means of constant electrical power supplied directly to a copper block containing the solution cell(s) and thermopile(s) assembled from commercially available thermoelectric units. Heat losses from the block are minimized by means of an adiabatic control system between the block and its jacket.

Major Findings: The basic noise level of the instrument, forty nanovolts peak-to-peak over a one hour period, is satisfactory. The calibration constant is 16 $W.V^{-1}$. It has been found that the steadiness of the base-line scanning depends upon the constancy of the power introduced into the block and on the quality of the temperature regulation maintained between the block and its jacket.

Significance to Bio Medical Research and the Program of the Institute: This instrument will be used to study phase transitions in biological macromolecules and cell membranes.

Proposed Course of Project: The calorimeter is presently undergoing a rigorous series of tests.

Serial Number: NIAMDD-LMB-12
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Thermodynamics of Monomer - Polynucleotide Complexes

Previous Serial Number: (none)

Principal Investigator: Philip D. Ross

Other Investigator: Robert L. Scruggs

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: To obtain a complete set of data on the system adenosine-polyribouidylic acid - salt - water in order to rigorously account for the calorimetric and equilibrium measurements of the binding of monomer to polymer.

Methods Employed: The binding isotherms are determined by: calorimetric titration, dialysis equilibrium, and spectrophotometric melting curves. The activities of the monomeric component and their dependence upon salt and monomer concentration and temperature are determined by vapor pressure osometry and heats of dilution.

Major Findings: There is a large (fifty per cent) discrepancy between the enthalpies of binding measured calorimetically and by equilibrium methods. This result is characteristic of a large number of monomeric-polynucleotide complexes that have been studied. Hill has recently given a thermodynamic treatment of this problem which can be applied to this system and should yield a completely consistent set of data thus unifying the diverse measurements that have been made. We are now in the process of gathering experimental data to perform this analysis.

Significance to Bio Medical Research and the Program of the Institute: Successful interpretation of these results will both clarify the significance of these types of measurements on polynucleotides systems and rigorously account for the observed properties of polynucleotide-mononucleotide complexes.

Proposed Course of Project: This work is being continued.

Serial No. NIAMDD-LMB-13
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: X-ray Diffraction Studies on Polyguanylic Acid

Previous Serial Number: (none)

Principal Investigator: Steven B. Zimmerman

Other Investigator: David R. Davies

Man Years

Total	1/4
Professional:	1/4
Other:	0

Project Description:

Objectives: To obtain structural information on poly G through x-ray fiber diffraction techniques.

Methods Employed: Standard techniques of biochemistry and x-ray diffraction.

Major Findings: Fibers of poly G giving interpretable x-ray diffraction patterns were prepared. There are relatively few data in the pattern because of the high symmetry of the structure. Based on several lines of evidence, we find the pattern best fits a four-stranded structure. Because of the great similarity between the patterns given by poly G and poly I, it seems quite likely that poly I also forms a similar four-stranded structure rather than the three-stranded structure proposed by Rich.

Significance to Bio-Medical Research and the Program of the Institute: Both normal and diseased states of tissues are controlled to an impressive extent by polynucleotides. Structural studies on polynucleotides may offer new insights into the functioning of these molecules. The current work constitutes the first relatively strong evidence for the existence of a new type of secondary structure, namely a four-stranded structure.

Proposed Course of Project: Further model-building and transform calculations will be needed to complete the poly G studies.

Serial No. NIAMDD-LMB -14
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project title: Enzymes from Mammalian Tissues which are Involved in the Repair or Replication of DNA.

Previous Serial Number: (none)

Principal Investigator: Steven B. Zimmerman

Other Investigator: Patricia McKinley

Man Years

Total:	3/4
Professional:	1/4
Other:	1/2

Project Description:

Objectives: To isolate and characterize several enzymes from mammalian sources which act on DNA, including polynucleotide kinase and polynucleotide ligase, in order to characterize them generally and in particular to test their interaction with DNA in various regions and types of chromatin.

Methods Employed: Standard techniques of biochemistry.

Major Findings: There are several reports in the literature of polynucleotide kinase activity in extracts of rat liver nuclei. We confirm this and have initiated purification of this activity; it is a stable activity and appears amenable for this purpose. The same initial steps which are used for the kinase purification (nuclei isolation and phosphocellulose chromatography) were used very satisfactorily for purification of polynucleotide ligase of this tissue in prior unpublished work by us and so we are proceding with the purification of both activities in joint fashion.

Significance to Bio-Medical Research and the Program of the Institute: It is not clear at present at what stage of the cell cycle enzymes of the type being studied here can get at their substrate, DNA, or even if most of the DNA is ever available. These and related enzymes are presumably responsible for maintaining the integrity of the DNA, yet the DNA is to a large extent covered with protein. The implications of unrepaired defects in DNA are manifold and have been suggested to be significant factors in many disease states as well as in aging and differentiation.

Proposed Course of Project: To pursue purification of these enzymes and characterize them, particularly with regard to their specificity for the nature of the DNA substrates and the effect of partial or total "masking" of the DNA by proteins as occurs in "active" and "inactive" or "open" and "closed" regions of chromatin.

Serial No. NIAMDD-LMB-15
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Aggregation of Polynucleotides at Acid pH.

Previous Serial Number: NIAMDD-LMB-12

Principal Investigator: Steven B. Zimmerman

Other Investigator: Patricia McKinley

Man Years

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives: To describe and better understand the mechanisms involved in the aggregation of polynucleotides at acid pH.

Methods Empolyed: Standard techniques of biochemistry and biophysics.

Major Findings: Polynucleotides precipitate at well defined pH values. These pH values are dependent upon both the base composition and the ionic strength of the medium. At low ionic strength, all the polymeric components in solution tend to precipitate together. The pH value at which such coprecipitation occurs can be predicted based upon a simple model which requires charge neutralization between the bases and phosphate residues for insolubility, *i.e.*, precipitation occurs at the net isoelectric point of the solution. Spectrophotometric pK values for some common nucleosides were determined for use in calculating the isoelectric points. At higher ionic strengths, there were instances of fractional precipitation. Also at higher ionic strengths, several homopolymers (poly A, poly G, and to a lesser extent, poly U) had a second zone of insolubility near neutral pH values.

Significance to Bio-Medical Research and the Program of this Institute: Interactions of polynucleotides with themselves and with other molecules are basic factors in the successful functioning of a cell. The current studies are one facet of such interactions and may extend understanding to related phenomena.

Proposed Course of Project: The experimental part of this project is completed. Preparation of the results for publication is being completed.

Publications: Steven B. Zimmerman and Norma F. Coleman, Biopolymers, 11 1943 (1972).

Serial No. NIAMDD-LMB-16
1. Laboratory of Molecular Biology
2. Section on Theoretical Mol. Biol.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Statistical Thermodynamics of Polynucleotide - Complementary Monomer Interactions

Previous Serial Number: NIAMD-LMB-20

Principal Investigator: Dr. T. L. Hill (8/12)

Other Investigators: Dr. Y. Chen (4/12), Dr. N. Saito (12/12)

Man Years:

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives: (a) To clarify the thermodynamic procedures which should be used in comparing optical, calorimetric, and binding measurements on these systems; and (b) to attempt to interpret these experiments in terms of a statistical mechanical model.

Major Findings: The thermodynamics of small systems has been applied to the binding of ligands on dilute polymer molecules in solution, and especially to the binding of complementary monomers and oligomers on polynucleotides. Topics emphasized are: binding isotherms, optical melting curves, calorimetric melting and mixing studies, role of the activity of the ligand, quasi phase transitions, the polymer phase diagram, and interrelations between these subjects. The one-dimensional Ising model (linear and circular) was used to illustrate some of the thermodynamics. The main object here has been to provide experimentalists in the field with the proper thermodynamic framework with which to analyze and compare their results. (Hill)

A quite general theory of aggregation in solution has been worked out. The main application we have in mind is to the stacking of bases, nucleosides, etc., but this is just a special case. The theory applies as well to protein aggregation, etc. The new feature is to show how the (a) relatively strong forces leading to aggregation and (b) other forces of interaction between aggregates (usually ignored) can both be included in a single formulation, without approximation. At the least, under (b), "hard" interactions between aggregates should be included in many cases (e.g., base stacking). (Hill and Chen)

Exact expressions have been derived for the distribution function of the end-to-end vector of a random coil chain under the restriction that the chain makes a definite number of turns around a fixed point (2-dimensional case) or a fixed straight line (3-dimensional case). The ring-closure probability and the mean square end-to-end distance have also been calculated. Applications are to the entanglement of polymer chains, for example in circular double stranded DNA. (Saito and Chen)

Significance to Biomedical Research and the Program of the Institute:

This project will contribute, through interpretation of experimental data, to our understanding of the forces involved in stabilizing polynucleotide and nucleic acid molecules in solution.

Publications:

Hill, Terrell L.: Thermodynamics of Ligand Binding on Dilute Polymer Molecules in Solution: Complimentary Monomers and Oligomers on Polynucleotides. Biopolymers 12, 257 (1973).

Hill, Terrell L. and Chen, Yi-der: Theory of Aggregation in Solution. I. General Equations and Application to the Stacking of Bases, Nucleosides, etc. Biopolymers (in press).

Saito, N. and Chen, Y.: Statistics of a Random Coil Chain in the Presence of a Point (2-dimensional case) or Line (3-dimensional case) obstacle. J. Chem. Phys. (in press).

Serial No. NIAMDD-LMB-17
1. Laboratory of Molecular Biology
2. Section on Theoretical Mol. Biol.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Ion transport across the nerve axon membrane

Previous Serial Number: NIAMD-LMB-19

Principal Investigator: Dr. T. L. Hill (4/12)

Other Investigators: Dr. Y. Chen (8/12)

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: To attempt to interpret experimental kinetic, steady-state, and noise data on K^+ (especially) and Na^+ transport across the nerve axon membrane in terms of a model in which the channels are protein complexes undergoing voltage-dependent conformational changes.

Major Findings: In recent experimental work, H. Fishman was able to remove the non-specific $1/f$ K^+ current noise and hence analyze the remaining $1/f^2$ noise which is associated with the specific K^+ channels. His results are very similar to what we predicted theoretically in a previous paper (last year's report). We have therefore been encouraged to extend our earlier work on noise considerably. We have come to realize the possible use of noise measurements in analyzing biochemical and biophysical kinetic systems generally. Hence our new work applies to any steady-state or equilibrium first-order kinetic scheme. (Chen and Hill)

We have extended earlier work on cooperative models of membrane ion transport to an exact Monte Carlo analysis of a 10 x 10 Ising System (with periodic boundary conditions). In these models, it is assumed that the K^+ channels interact with each other. We show that, for practical purposes, 10 x 10 (periodic boundary conditions) is an "infinite" Ising system. The point of this work is to demonstrate that models of this type (still being pursued by G. Adam and others) cannot account for basic experimental facts. In particular, the K^+ current curves, in voltage clamp depolarizations, do not show the observed early induction behavior, or the observed superposition. (Chen and Hill)

Significance to Biomedical Research and the Program of the Institute:

An understanding of the operation, at the molecular level, of a normal nerve membrane is prerequisite to a rational attack on various neurological and neuromuscular abnormalities.

Publications:

Chen, Yi-der and Hill, Terrell L.: Fluctuations and Noise in Kinetic Systems; Application to K^+ Channels in the Squid Axon. Biophysical J. (in press).

Chen, Yi-der and Hill, Terrell L.: On the Theory of Ion Transport Across the Nerve Membrane. VII. Cooperativity Between Channels of a Large Square Lattice. Proc. Nat. Acad. Sci. 70, 62 (1973).

Serial No. NIAMDD-LMB-18
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Genetics and structure of the oncogenic virus, SV40

Previous Serial Number: NIAMD-LMB-3

Principal Investigator: Robert G. Martin (12/12)

Other Investigators: Dr. Peter McCann (12/12), Dr. Rein Saral (12/12),
Dr. Janice Y. Chou (12/12), Dr. Jesus Avila (12/12),
Dr. Robert D. Brown (9/12)

Collaborators: Dr. O. Wesley McBride (CLB)

Man Years:

Total:	5-8/12
Professional:	5-8/12
Other:	0

Project Description:

Objective: To analyze the genetic structure of the oncogenic virus, SV40, through the isolation of conditional lethal mutants. Interaction between host functions and viral replication will also be investigated.

Major Findings: (a) Characterization of the SV40 DNA core complex (McCann and Martin) has been undertaken. A nucleoprotein complex can be isolated from SV40 upon alkaline disruption of virions. The complex, unlike histone DNA complexes of chromatin is completely susceptible to nuclease degradation. However, when the protein in the complex is cross linked to the DNA by formaldehyde, 1% of the DNA becomes resistant to the action of nuclease. By isolation of the resistant material and analysis by DNA renaturation studies with appropriate controls it has been shown that the protein is randomly associated with the DNA,making it unlikely that the DNA protein complex plays any role in the replication or transformation processes of the virus.

(b) A number of temperature sensitive mutants of the virus have been isolated. (Chou and Martin). Unfortunately this work is extremely time consuming and tedious but several mutants have now been isolated, conditions for mutagenesis have been fully developed and it should be possible to obtain approximately 100 such mutants in the coming year. Assuming random mutagenesis this number of mutants should be sufficient to saturate the genetic map. Only preliminary characterization of these mutants has been undertaken thus far, although there appear to be both early (prior to DNA replication) and late (post DNA replication) mutants represented. In addition a slant gel technique has been developed for the rapid determination of complementation groups.

(c) Preliminary characterization of coat mutants (Avila, Brown, Martin). Although approximately 40 "late" mutants of SV40 are available (these mutants presumably have a defect in one of the viral capsid proteins) and there are known to be two major capsid proteins, all of the late mutants appear to fall into one complementation group. This means either that all of the mutants have a defect in only one of the capsid proteins or that there is something peculiar about the complementation process. To determine which of these possibilities is correct attempts have been undertaken to purify the capsid proteins from wild type and mutant viruses in order to determine which capsid protein is defective in the various temperature sensitive mutants. At this point, procedures for isolation of the capsid protein radio labelled in sufficient quantity have been worked out, but determination of the defects has not yet been accomplished.

(d) Orientation of integrated SV40 (Saral & Martin). In order to determine the orientation of SV40 in the integrated state in mouse transformed cell lines, a number of enzymes and substrates had to be purified and conditions for their use determined. We have isolated large quantities of Bromodeoxyuridine labelled SV40 DNA, R_1 restriction enzyme and terminal transferase for this study.

(e) Transformation of Chinese hamster cells by SV40 (Martin & McBride). A number of clones of SV40 transformed Chinese hamster cells have been isolated. Chromosomes from these lines will be fractionated and reannealing studies carried out to determine whether SV40 always integrates into the same chromosomes or whether integration can occur in different chromosomes.

(f) Fractionation of chromosomes (Avila, Martin & McBride). In order to facilitate the previous study, high resolution fractionation of chromosomes is being attempted. The proposed procedure involves the use of affinity chromatography. To this end, agarose of appropriate porosity has been synthesized which allows the uninhibited flow of chromosomes through a column. Qunicrine mustard has now been attached to this matrix.

Significance to Bio-medical Research and the Program of the Institute:

The significance of these projects is that they may lead to some insights into carcinogenesis and other metabolic diseases.

Publications:

Kidwell, William R., Saral, Rein, Martin, Robert G., and Ozer, Harvey L: Characterization of an endonuclease associated with simian virus 40 virions. J. Virology 10: 410-416, 1972.

Chou, Janice Y. and Martin, Robert G.: Purine phosphoribosyltransferases of *Salmonella typhimurium*. J. Bacteriology 112: 1010-1013, 1972.

Serial No. NIAMDD-LMB -19
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The involvement of bacterial functions in biological processes caused by the infection of bacteriophage and bacterial plasmids

Previous Serial Number: NIAMDD-LMB-8

Principal Investigator: Toshiya Takano

Other Investigators: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: An understanding of the involvement of bacterial functions in the replication of bacterial viruses. Specifically, attention has been focused on:

a) Molecular genetic studies on the process of the morphogenesis of the head of bacteriophages.

b) Investigation of a bacterial mutant affecting both plasmid formation and cellular division.

c) The biochemical genetic studies of the functions of a new type of regulator genes, which has pleiotropic controls on several different operons.

d) Genetic and biophysical studies on the homology of the deoxyribonucleic acid (DNA) of bacterial plasmids.

Major Findings: a) 1) The processes of morphogenesis of bacteriophage heads were studied. A bacterial mutant that restricts the growth of phage T4 by a block of head formation, named "mop" (morphogenesis of phage), has been isolated. The mutation is located at 83 minutes on the K12 chromosome map. Mutant phages, that can grow on the bacterial mutant were also isolated and the mapping of these phage mutants shows that these mutations are in gene 31 of T4. It is concluded, therefore, that there is a process in a very early stage of the morphogenesis of phage heads, that is catalysed by a bacterial factor controlled by mop gene, and the defect of the factor is compensated by the mutation of gene 31 of phage T4. This bacterial mutant also restricts phage λ and ϕ80, so the bacterial function may be involved generally in the formation of phage heads.

2) Two independent findings showed that the lesion of the mop⁻ bacteria is on the membrane; the fourth major peak of the protein subunits of the bacterial membrane on an acrylamide gel electrophoresis is missing in the bacterial mutant. The permeability of such tri-peptides as tri-serine and tri-tyrosine is blocked in the mutant, so that the mop⁻ strain is resistant to these agents that are very toxic to normal bacterial growth. The block of the morphogenesis of phage head causes the random aggregation of the precursor protein of the head on the bacterial membrane of the mutant, mop. Therefore, it is concluded that the mop gene controls one of the proteins of the bacterial membrane, and the bacterial membrane plays an important role in the morphogenesis of the precursors of phage heads.

3) In order to study further the mechanism of the morphogenesis of phage head, *in vitro* complementation between gene 23 and 31 products has been studied. Many other investigators had previously tried unsuccessfully to make this system work. The *in vitro* complementation was performed with a mixture of the two extracts of the cultures of non-supressible bacteria infected by T4 mutants: one was infected by an amber mutant of T4 gene 31, which donates the product of gene 23, a major precursor of head protein, but lacks the product of gene 31; the other was infected by an amber mutant of T4 gene 23, which donates the product of gene 31, a catalytic, morphopoietic factor, but lacks the precursor. After incubation, the increase of plaque-forming units was assayed. Successful complementation was observed, only when the extract from the bacteria infected by a T4 mutant that can overcome the block of the bacterial mop mutation, was used as a donor of the product of gene 31. No complementation was observed, however, with the extract from bacteria infected just with an amber mutant of gene 23. Besides the specific donor for the gene 31 donor, the positive complementation required a membrane fraction of the cultures and adenosine triphosphate, and was inhibited by the addition of pancreatic deoxyribonuclease I (5 μg/ml). The DNA of most phage particles formed in these *in vitro* experiments came from the extract of the bacterial culture infected by the phage mutant that can overcome the block of the mop mutation. The reasons for this successful complementation are as follows: one is, as shown above, that bacterial membrane fractions were considered as an important factor in the process of the head morphogenesis; the other is that an appropriate mutant of T4, was used as a donor of the product of gene 31, in whose infected bacteria the phage precursor DNA and the product of gene 31 seemed to be intactly sitting on the membrane fraction. Contrariwise the product of the usual gene 31 could not form an intact structural arrangement of phage DNA and bacterial membrane in the absence of the product of gene 23, resulting in irreversible aggregation of the precursors on the membrane fraction. The further characterization of the *in vitro* reaction and the purification of the products of gene 31 and 23 are under way.

b) Bacteriophage P1 can lysogenize bacteria and replicates as a plasmid synchronous with the cellular division cycle, without integration into the host chromosome. In a bacterial mutant, lon$^-$, P1 cannot lysogenize and always gets into a vegetative growth cycle (T. Takano, Proc. Nat. Acad. Sci., 68: 1496 [1971]). A temperature-sensitive lon$^-$ mutant has been isolated. Prophage P1, that was lysogenized at 30°C in the lon ts strain, was induced by a shift-up of the temperature to 40°C. These results suggest that the product of the lon gene acts like a repressor of P1 prophage, and is needed for maintenance of synchronous replication of P1 prophage.

On the other hand, mutants of phage P1, that can lysogenize even on the lon$^-$ bacteria, were isolated and classified by complementation tests for lysogenization on a lon$^-$ strain. One, which was cis-dominant, could lysogenize on a lon$^-$ strain as frequently as on a lon$^+$ bacteria, but could not help the lysogenization of wild type P1 on a lon$^-$ strain in mixed infection. The other, which was trans-dominant, could help the lysogenization of the wild type phage. The former might be a mutation of the site which responds to the bacterial repressor, the lon$^+$ factor, and which changed the mutation to be constitutively repressed. The latter might be explained by a quantitative or qualitative mutation of a diffusible factor, such as another repressor controlled by a gene of phage P1, which became constitutively repressed even in the absence of the lon$^+$ function.

c) The lon$^-$ mutant is also known to be defective in septum formation in cellular division and to overproduce cellular polysaccharides (see ref. Proc. Nat. Acad. Sci.). In order to investigate the mechanism of the unique regulation by the lon gene that represses several independent bacterial gene functions involved in the processes of cellular division cycle, I studied the regulation of one of the loci involved in polysaccharide synthesis, the galactose operon, by lon and another regulator gene, galR. Defects of either regulator gene cause the constitutive derepression of the production of the enzymes controlled by gal operon. I constructed a bacterial strain carrying both lon$^-$ and a super-repressible mutation of galR (galR^S). The activities of all three enzymes of the gal operon were determined on the extracts of the cultures incubated at various temperatures. The effects of the lon$^-$ and galR^S mutations on these enzyme activities were just additive throughout the temperature range from 25 to 40°C. This suggests that the lon$^+$ gene might act just as another but independent repressor of the gal operon.

d) P1 is unique because it is a plasmid that has another life cycle as a vegetative virus. All other known plasmids, like F, R, or Colinogenic factors have only the cycle as a plasmid. In order to study the genetic and evolutionary relationships between these plasmids and P1-like bacteriophages, I made hybrids between P1 and R factors. These hybrids were as infective as the parental phage P1, and also carried the resistance markers to such drugs as kanamycin or ampicillin. These results suggest that P1 and other plasmids carry some genetically homologous regions and are evolutionarily related. The genetic and biophysical analysis of this homology is under investigation.

Significance to bio-medical research and the program of the institute: Viruses induce a series of new biological processes in the infected host cells. To understand the relationships between the host and viral functions at a molecular and enzymological level, is an important goal for the future control of these viral processes including the infection and oncogenesis by animal viruses. Specifically, bacteria and their bacterial viruses give simpler and quicker approaches to these goals than mammalian systems, and will provide models for these processes in mammalian systems.

Publications:

Takano, T. and Kakeduda, T.: Involvement of bacterial factor in the morphogenesis of bacteriophage capsid. Nature New Biology, 329: 34-37, 1972.

Takano, T.: Current review on lysogenic phages, 1972. Protein, Nucleic Acid and Enzymes (Tokyo) 17: 77-78, 1972.

Takano, T.: A bacterial function required for plasmid formation by P1 and λNNpGal8. Abstracts of the Cold Spring Harbor Bacteriophage Meeting, 69, 1972.

Serial No. NIAMDD-LMB-20
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Genetics and physiology of bacteriophage P1

Previous Serial Number: NIAMD-LMB-6

Principal Investigator: J. L. Rosner (12/12)

Collaborators: Singer, M. (ALBM); LeBlanc, D. (ALBM)

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: An understanding of the mechanisms which regulate vegetative growth, plasmid formation, transduction and gene expression by the temperate bacteriophage P1. Special attention has been devoted to:

a) The isolation and characterization of P1 prophages which carry bacterial genes and which are deleted for P1 genes.

b) The P1 modification-restriction system.

Major Findings: a) Variants of bacteriophage P1 which carry *Escherichia coli pro* genes (encoding the proline-biosynthesis enzymes) have been isolated and partially characterized. Pro^- rec^- (recombination deficient) bacteria were infected with P1 which had been grown on pro^+ bacteria and pro^+ transductants selected. The rec^- mutation eliminated generalized transductants as a source of pro^+ transduction. About 10^{-9} to 10^{-10} of the P1 were able to transduce the pro^- rec^- bacteria to pro^+, because the *pro* genes were linked to the P1 replicon. A series of independently isolated P1 *pro* variants were studied and found to fall into two categories: those which are P1 defective (P1d*pro*) i.e. have lost some P1 genes and those which carry a full complement of P1 genes (P1p*pro*). The P1d*pro* strains all have deletions of P1 genes beginning at a particular region of the P1 chromosome but vary in the point where the deletions terminate. One small portion of the P1 chromosome is invariably present in the P1d*pro* strains suggesting that this region is where the *pro* genes are linked to the P1 chromosome and, further, this region is sufficient to ensure the replication and segregation of the P1 prophage in coordination with that of the chromosome. It is of interest that the strains carrying this region only are neither P1 immune nor excluded by the presence of a normal P1 prophage.

The genetic map of P1, constructed from the results of vegetative crosses, is linear. The regions deleted in the P1dpro strains include both ends of this map. The simplest interpretation of the observations is that the deletions arise from circular P1 molecules, in accord with the fact that P1 can circularize its DNA.

Lysates prepared by infecting the P1dpro strains with a P1 virulent mutant have high frequency transducing (HFT) activity for pro. The pro$^+$ transductants obtained from infection with these lysates are again defective.

The P1ppro strains appear to contain the pro genes linked to a full complement of P1 genes. When induced, they produce HFT lysates. However, the pro$^+$ transductants obtained from this lysate are defective and the non-defective particles in the lysate do not contain pro$^+$ activity. This is understandable in terms of the "head-full" mechanism whereby P1 capsids incorporate a fixed length of DNA. The P1ppro genome is too large to be encapsidated but portions of it can be. The portions lacking some P1 genes would be P1dpro while those lacking the pro genes would be normal P1.

b) The bacteriophage P1 modification-restriction system. Previous work in this laboratory demonstrated that certain mutants of P1 which are unable to lysogenize E. coli are deficient in the P1 modification (methylase) function. Further experiments have supported the hypothesis that these modification-deficient mutants are restriction (endonuclease) proficient. In collaboration with Drs. Singer and LeBlanc, the methylase and nuclease enzymes elaborated by wild type and mutant strains are being assayed to test this hypothesis directly. In addition, these enzymes, which are believed to form a complex, will be isolated and how they interact with each other will be studied.

Significance to bio-medical research and the program of the institute:

Knowledge of the interaction of virus and host cell is important for an understanding of a variety of diseases. Insofar as this research contributes to our understanding of these interactions as they occur in bacteria, it will have significance to the objectives of this institute.

Restriction and modification is a mechanism whereby cells differentiate between foreign and native DNA. A better understanding of the bacterial systems may facilitate the detection of similar systems in higher organisms. Of particular interest would be the possibility that certain viral diseases are manifestations of restriction-modification systems. The mechanism whereby restriction and modification enzymes recognize particular sequences of DNA is of fundamental importance.

Proposed course of project:

a) The DNA of the various P1pro prophages will be isolated and sized by sedimentation characteristics and electron microscopy. Heteroduplexes made from the P1pro DNA and normal P1 DNA will be constructed and visualized by electron microscopy. These studies should permit a determination of how much bacterial and P1 DNA is present in each strain and to correlate the physical and genetic maps of P1.

b) The course of the work on the P1 restriction-modification system has been indicated.

Publications:

Hidalgo, C. and Rosner, J. L.: Methylation of bacteriophage P1 DNA. Virology 50: 280-281, 1972.

Rosner, J. L.: Modification-deficient mutants of bacteriophage P1. I. Restriction by P1 cryptic lysogens. Virology 52: 213-222, 1973.

deCrombrugghe, B., Shaw, W. V., Rosner, J. L. and Pastan, I.: Stimulation by cyclic AMP and ppGpp of chloramphenicol acetyl transferase synthesis. Nature New Biology 241: 237-239, 1973.

Annual Report of the
Mathematical Research Branch
National Institute of Arthritis, Metabolism, and Digestive Diseases

Let m(x) denote the maximum entry in the vector x. We call a matrix K maximum increasing if $m(Kx) \geq m(x)$, a concept due to Ky Fan. It has been shown that exp(At) is the inverse of a maximum increasing matrix if and only if the off diagonal entries of A are nonnegative and its row sums are nonpositive. This shows that if $\dot{x} = Ax$ $x(0) = x^0$, then $m(x) \leq m(x^0)$ for all t. In particular the specific activity in any compartment of a compartmental system cannot exceed the maximum initial value. More, only linear systems with the characteristics of specific activities enjoy this property. (Dr. J. Hearon)

A general characterization has been obtained for flow patterns in steady-state capillary networks which maximize substrate extraction (Microvascular Research, 5(2):180-198, 1973). (Dr. J. M. Gonzalez-Fernandez and Mrs. S. Atta)

Work on the transient problem of oxygen extraction in skeletal muscle has continued. By taking account of the histological structure of skeletal muscle a mathematical model for the transport-consumption of oxygen has been formulated incorporating the following features: At the tissue-capillary level the geometry of Krogh's concurrent model; a Michaelis-Menten type consumption in tissue; reversible binding with myoglobin; at the arterial venous level, counter-flow exchange and diffusion into tissue as well as competition between arteries and capillaries in diffusional exchange; at the capillary-capillary level countercurrent exchange is allowed. A computer program for numerical solutions has been written. (Dr. J. M. Gonzalez-Fernandez and Mrs. S. Atta)

A new algorithm has been incorporated into the iterative method for solution of the linear systems arising in numerical solution of elliptic partial differential equations. The algorithm maximizes the rate of convergence. A vector of over relaxation factors is maximized in such a way as to reduce the norm of the matrix for the iterative process together with techniques which ensure monotone convergence. (Mrs. S. Atta)

Work has continued on the systematic construction, analysis and testing of a group of interrelated models which account for many different sequences of events in a dendritic neuron. A great deal of material, much of it original, is being organized in preparation of a chapter for the new Handbook of the Nervous System. Computational exploration of the behavior of a model for active nerve membrane in regions of changing geometry is being carried out (in collaboration with Dr. S. Goldstein; see below). These results draw heavily upon studies reported here in previous years. Research on branch input resistance and dendritic spine calculations (in part described in previous reports) has continued (in collaboration with Dr. John Rinzel, Courant Institute) with the first of a substantial series of papers being in press. (Dr. W. Rall)

A theoretical study of the influence of the geometric properties of a neuron upon its primary electrical properties has been carried out (in collaboration with Dr. W. Rall). It has been shown that the velocity of propagation of the action potential is strongly influenced by the diameter of the axon. The velocity increases proportionally to the square root of the diameter but decreases as the rate of change of diameter with distance increases. The effect of taper on velocity is thus determined by the balance of these two factors. The sum of 3/2's power of branch diameters determines the change in velocity in the region of a branch point. The study is also illuminating in terms of such items as safety factor, threshold and shape-velocity relations. (Dr. S. Goldstein)

A new computer-assisted system for recording the "present illness" section of the medical history has been devised and given preliminary testing. The computer presents a list of symptoms referable to a (physician chosen) organ system. Given a symptom its characteristics may be modified by a second computer presented list. Information is recorded during the interview and is rearranged chronologically by the computer before the history is printed. (Dr. S. Goldstein)

Studies have been carried out on the response of single cells to extracellular (electrical) stimulation. The exact solution for a spherical cell in a uniform applied field in a large volume conductor shows that over a broad physiological range cells behave as if the membrane were a perfect insulator. It has been shown by numerical solution that the presence of a low resistance patch results in a shift of the isopotential value of the interior towards the exterior value just outside the patch. This result suggests the availability to the nervous system of a method of modulating the effects of local fields. Theorems have been proved which set the maximum and minimum exterior surface potential as upper and lower bounds for the interior potential in the static case and which set an upper bound for the magnitude of the internal electric field. (Dr. M. Klee)

Work on the "central core" model of the renal counterflow system has continued to expand. An initial paper describing the model has appeared (Kidney International, 2:85-94, 1972). Two papers on the theoretical details have been accepted for publication (to appear in Biophys. J., June, 1973). A paper on the numerical analysis (in collaboration with R. Mejia, DCRT) has been accepted for presentation (and publication) at the 1973 Summer Computer Simulation Conference in Montreal. Various aspects of this work have been presented at the IV International Biophysics Congress, Moscow; the V International Nephrology Congress, Mexico City; the annual meeting of the Biophysics Society; and at the Houston Symposium on Biomathematics and Computer Science. (Dr. J. Stephenson, NHLI, Associate Member, MRB)

Considerable effort has been directed toward numerical analysis of the differential equations describing the various models. In part it has been possible to integrate the DE's by using standard techniques and existing programs, but the character of the boundary conditions and large values of certain parameters occurring in the equations strictly limit the usefulness of this approach. Adaptation of "stiff" system techniques has greatly increased the efficiency of integration. (Dr. J. Stephenson, NHLI, Associate

Member, MRB; Mr. Raymond Mejia, DCRT, and Dr. B. Hubbard, University of Maryland)

Earlier work on the transient behavior of counterflow systems has been written up for publication (Bull. Math. Biophys., in press). This work has been extended. In particular, it has been found feasible to analyze some systems by numerical inversion of the Laplace transforms of the system. (Dr. J. Stephenson, NHLI, Associate Member, MRB and Dr. Kenny Crump, Louisiana Polytechnical Institute)

Studies of reconstruction (of a 3-dimensional object from a finite collection of projections) techniques have continued. It has been shown that one version of ART (cf. Gordon, these reports, 1972) converges upon the solution of minimum variance. An algorithm has been devised for locating artifacts in reconstructions from a small number of projections. An extensive review and comparative study of reconstruction techniques has been completed. (Dr. R. Gordon with the collaboration of Dr. Gabor T. Herman, SUNY, Buffalo)

Computer simulation of the changes in shape of the neural plate (newt) have continued. The program represents the plate as a disc comprised of hexagonally packed regions. Each region is assigned a height function. The program shrinks the regions under constant volume constraint and repacks the regions in such a way as to preserve continuity of the cell sheet so represented. It has been shown that two forces are required, nonuniform decrease in cell area due to increased height and elongation of the underlying notochord, and that extensive slippage and changes in cell-cell contact occur in the neural plate. (Dr. R. Gordon, in collaboration with Prof. A. G. Jacobson, University of Texas)

Serial No. NIAMDD, ODIR, MRB-1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mathematics of kinetics and reaction-transport systems

Previous Serial Number: SAME

Principal Investigator: John Z. Hearon

Man Years:

Total: 1.3
Professional: 1
Others: .3

Project Description:

Objectives: The objectives of this research remain substantially as stated in previous reports.

Major Findings: In addition to consultative and collaborative activity, efforts during the past year were concerned with the following work:

Maximum Increasing Matrices.

Following Ky Fan, we say that a matrix K is maximum increasing if $m(Kx) \geq m(x)$ for all x, where m(y) denotes the maximum entry in the vector y. It is known that K is maximum increasing if and only if K^{-1} exists, is non-negative and has row sums not greater than one. It has been shown that if A is a matrix such that $a_{ij} \geq 0$, $i \neq j$, and $\Sigma_j a_{ij} \leq 0$ then exp(At) is, for all t, the inverse of a maximum increasing matrix. In fact exp(-At) is maximum increasing only if A has the above properties. The proof is rather simple. It is well known that $\exp(At) \geq 0$ if and only if $a_{ij} \geq 0$. Thus we have only to prove that $f = \exp(At)u \leq u$, where $u^*=[1,1,\ldots,1]$, if and only if $Au \leq 0$.

Clearly $f(0) = u$ and if $Au \leq 0$ then $\dot{f}(t) \leq 0$ for all t. Conversely if one component of Au is strictly positive then some f_k is initially unity and initially increasing and must exceed unity.

The importance of this theorem is that the solution of $\dot{x} = Ax$, $x(0) = x^o$ is $x(t) = \exp(At)x^o$ and from the theorem it follows that $m(x) \leq m(x^o)$ for all $t \geq 0$. Thus no component $x_i(t)$ can at any time exceed the maximum of $x_1^o, x_2^o, \ldots, x_n^o$. This applies in particular to the case that x_i is the specific activity in the i^{th} compartment of a stationary system. But the result also shows that $m(x) \leq m(x^o)$ holds only if A has the properties $a_{ij} \leq 0$ and $Au \leq u$.

Washout and Residence Times.

It has been shown that for an arbitrary compartmental system with matrix A and initial vector c, the mean residence time $\bar{t}$ is

$$\bar{t} \quad = \quad -u^*A\bar{c}^{1}/u^*c \qquad (1)$$

where u is defined above. We now define the quantities

$$\tau_i \quad = \quad -u^*A\bar{u}_i^{1} \qquad i=1,2,\ldots,n \qquad (2)$$

where u_i has 1 in the i^{th} position and zeroes elsewhere. From (1) and (2) it follows that τ_i is the mean time when all tracer is initially in the i^{th} compartment, and that

$$\bar{t} \quad = \quad \Sigma\, w_i\, \tau_i \qquad (3)$$

where $w_i = c_i/\Sigma\, c_i$. Thus $\bar{t}$ is the weighted mean of the residence times $\tau_1, \tau_2, \ldots, \tau_n$. The washout function is

$$u^*x \quad \equiv \quad g = -u^*\, A^{-1}\, e^{At} v \qquad (4)$$

and from (4)

$$g(0) = (\Sigma \tau_i w_i')(u^*v)$$

$$= - \dot{g}(0) \bar{t}_s$$

where $w_i' = v_i/\Sigma v_i$ and $\bar{t}_s$ is the mean residence time under input vector v. Thus the initial value and slope of g suffice for an estimate of $\bar{t}_s$.

Matrix Deflation.

A matrix deflation is any procedure whereby given k roots of a matrix the remaining ν=n-k roots are to be found on the roots of a matrix of order ν. The following applies to any conservative linear system but we state it in general terms. Let K be given and partitioned as

$$K = \begin{pmatrix} a & r^* \\ c & A \end{pmatrix}$$

where A is of order n-1, r and c columns of length n-1 and $a=k_{11}$. Then we have proved:

If $w^*K = \mu w^*$ and λ is an r-fold root of K then λ is an r-fold root of $A-cw^*=B$. Further, the Jordon chain structure of λ as a root of K and as a root of B is the same. Indeed the chain of vectors of K can be reconstructed from those of B.

Publications:

Hearon, J. Z.: Compartmental matrices with single root and nonnegative nilpotent matrices. Math. Biosci. 14: 135-142, 1972.

Hearon, J. Z.: Residence times in compartmental systems and the moments of a certain distribution. Math. Biosci. 15: 69-77, 1972.

Hearon, J. Z. and London, W. P.: Path lengths and initial derivatives in arbitrary and Hessenberg compartmental systems. Math. Biosci. 14: 121-134, 1972.

London, W. P. and Hearon, J. Z.: Estimation of the number of precursors in a sequence of first order reactions. Math. Biosci. 14: 281-295, 1972.

Serial No. NIAMDD, ODIR, MRB-2
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mathematical formulation and analysis of problems relevant to experimental neurophysiology

Previous Serial Number: SAME

Principal Investigator: Wilfrid Rall

Other Investigators: Steven Goldstein

Man Years:

Total:	1.3
Professional:	1.0
Others:	.3

Project Description:

Over the past several years, this project has been concerned with constructing, developing and testing a group of interrelated mathematical models. This group of models provides a theory that can account for many different sequences of events in a dendritic neuron, and in certain populations of such neurons. Computational experiments performed with these models provide theoretical predictions that have been compared with the results of neurophysiological experiments and neuroanatomical observations. Also, insights have been obtained, which have led to new hypotheses, new interpretations, and to the design of new experiments.

1. Core conductor theory and cable properties of neurons. Literature research, organization of material and considerable writing effort are being devoted to preparation of a chapter for the new Handbook of the Nervous System, Volume I, Cellular Biology of Neurons, that is now in preparation for The American Physiological Society.

2. Computational exploration of the behavior of a mathematical model of active nerve membrane properties in regions of changing geometry (in collaboration with Dr. Steven Goldstein). This study is yielding interesting new results regarding the factor of safety in action potential propagation in regions where there is taper, or where branches arise, or where there is some other departure from uniform cylindrical conditions. It depends upon the results of earlier studies of the present project (i.e., the invention

and previous exploration of a useful mathematical model for active membrane, and the mathematical treatment of taper and branching). New results increase our understanding of conditions influencing propagation velocity and propagation failure. Dr. Goldstein and I are preparing some of these results for publication.

3. Branch input resistance and steady attenuation for input to one branch of a dendritic neuron model; also dendritic spine calculations and implications (with John Rinzel, formerly DCRT, now at the Courant Institute for Mathematical Sciences, New York University). This research has been described in previous reports. The first of several rather substantial papers arising from this collaboration has been accepted for publication in the Biophysical Journal. Final drafts of several subsequent papers have been delayed, and will probably not be completed until the next fiscal year.

Advisory Councils:

Central Council of IBRO (International Brain Research Organization, affiliated with UNESCO); 1968-1973.

Committee on Brain Sciences, National Research Council; 1968-1973.

National Council of the Society for Neuroscience; 1970-1973.

USA National Committee for IBRO, National Research Council; 1972-.

Publication:

Rall, W. and Rinzel, J.: Branch input resistance and steady attenuation for input to one branch of a dendritic neuron model. Biophys. J. (In press).

Serial No. NIAMDD, ODIR, MRB-3
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mathematical description of the transport-chemical reaction kinetics of substances in the blood-capillary-tissue complex

Previous Serial Number: SAME

Principal Investigator: Jose M. Gonzalez-Fernandez

Other Investigators: Susie E. Atta

Man Years:

Total:	2.3
Professional:	2
Others:	.3

Project Description:

Objectives: The broad objectives of this research remain substantially the same as the ones stated in the previous reports.

Major Findings:

Steady state:

A paper containing a general characterization for the flow patterns in capillary networks that yield a maximal substrate extraction has been published.

Nonsteady state:

The work on nonsteady state extraction of oxygen in skeletal muscle has been continued. By taking into account the histological properties of the capillary bed and small arteries and veins in the skeletal muscle, a description for the transport-consumption of oxygen has been formalized in a mathematical model. A computer program for the numerical solutions of the equations has been written. At the capillary-tissue level the formulation uses the geometry of Krogh's concurrent flow model. A Michaelis-Menten oxygen consumption at the motochondria, as well as the reversible combination of O_2 and Mb are incorporated. At the artery-vein level: 1) a counterflow exchange 2) a nutritional diffusion of O_2 into the tissues and 3) a competition of arteries and capillaries diffusional exchange are incorporated. At the capillary-capillary level an amount of countercurrent exchange is allowed. The numerical results obtained appear to describe the experimental results of oxygen extraction (Stainsby and Otis) in rest and exercise, the counterflow O_2 shunting (Duling and Berne) and the lack of marked influence on the tissue pO_2 of high oxygen arterial concentration (Whalen).

Numerical Analysis:

A new algorithm that maximizes the rate of convergence has been incorporated to the point successive iterative process for solving linear algebraic equations that arise in the numerical solution of elliptic partial differential equations by finite difference methods. A vector of over-relaxation factors are maximized by techniques that will reduce the norm of the matrix for the iterative process together with techniques that will ensure monotone convergence.

It is shown that the iterative process for a certain class of matrices converges more rapidly with a maximized vector of under-relaxation factors. It is shown that for two other classes of matrices more rapid convergence is obtained for a relaxation factor equal to one.

A manuscript will be written and submitted for publication in a numerical analysis journal.

Publications:

Gonzalez-Fernandez, J. M. and Atta, S. E.: Maximal substrate transport in capillary networks. Microvascular Research 5(2): 180-198, 1973.

Serial No. NIAMDD, ODIR, MRB-4
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Applications of electric field theory to cellular physiology

Previous Serial Number: NONE

Principal Investigator: Maurice Klee

Man Years:

Total:	1.0
Professional:	1.0
Others:	0

Project Description:

Objectives: To obtain an understanding of the electrical behavior of nerve and muscle in terms of three dimensional electric field theory.

Methods: Finite difference and integral equation numerical techniques are used to calculate the electric potential distributions within and surrounding single cells. Publications describing these methods appeared this year (see below).

To stimulate the use of numerical techniques a seminar was organized devoted to the systematic study of the numerical solution of partial differential equations. Participants from various institutes presented weekly lectures over a period of five months.

Major Findings and Significance: The emphasis during the past year of this project has been on determining the response of single cells to extracellular stimulation.

Using the analytic solution for a spherical cell in an applied uniform field, it was shown, within a broad physiological range, that cells behave during extracellular stimulation in a large volume conductor as if they had a perfect insulator for a membrane. This result allows one to replace the cell membrane by a perfect insulator in building physical models of complex cells or in using analytic solutions of boundary value problems. Also from this study of a spherical cell it was shown that the phase of the induced intracellular electric field is essentially identical with the phase of the membrane admittance. Experimentally, this result can be used in distinguishing between a simple RC membrane, a two-time-constant membrane, and a constant phase admittance membrane.

Using the numerical techniques discussed in Methods, it was shown that the presence, during extracellular stimulation, of a low resistance patch, such as produced by a synaptic event, in an otherwise high resistance membrane causes a shift in the isopotential value of the intracellular media towards the value of potential just outside the patch. This result implies that the response of a cell to extracellular stimulation depends upon the present state of synaptic input to the cell being stimulated and represents a method available to the nervous system of modulating the effects upon a cell of local electric fields. Experimentally, this shift in the isopotential value of the intracellular media can be used to measure the conductance change which occurs at a synapse.

Using basic theorems of vector calculus, two properties of the intracellular potentials and electric fields produced by static extracellular stimulation were obtained for arbitrarily shaped cells. First, the values of intracellular potential were shown to be bounded by the maximum and minimum values of extracellular potential on the surface of the cell. Second, the volume average of the magnitude of intracellular electric field was shown to have an upper bound given by the ratio of the magnitude of the largest extracellular potential difference on the surface of the cell to a generalized length constant λ:

$$\lambda = \left(\frac{\sigma_{intra} V_{cell}}{\sigma_{memb} A_{cell}} \right)^{\frac{1}{2}}$$

where V_{cell} and A_{cell} are the volume and surface area of the cell, σ_{intra} is the intracellular conductivity, and σ_{memb} is the membrane conductivity. The upper bound on the volume average of the magnitude of intracellular electric field serves as an estimate for intracellular isopotentiality and the generalized length constant allows one to electrically describe arbitrarily shaped cells.

Proposed Course: One further numerical experiment on the response of single cells to extracellular stimulation is planned; namely, the role of cell shape will be studied by examining the behavior of oblate and prolate spheroidal shaped cells in an applied uniform field. With the completion of this study, the emphasis of the project will shift to an examination of the three dimensional electric fields produced by the synaptic events of nerve and muscle.

Publications:

Klee, M. and Plonsey, R.: Finite difference solution for biopotentials of axially symmetric cells. Biophys. J. 12: 1661-1675, 1972.

Klee, M. and Plonsey, R.: Integral equation solution for biopotentials of single cells. Biophys. J. 12: 1676-1685, 1972.

Klee, M.: Intracellular biopotentials during static extracellular stimulation. Biophys. J. (In press).

Serial No. NIAMDD, ODIR, MRB-5
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mathematical analysis of kinetic problems in biochemistry

Previous Serial Number: SAME

Principal Investigator: Wayne P. London

Man Years:

Total:	.1
Professional:	.1
Others:	0

Project Description:

Objectives: To develop mathematical models of the kinetics of biochemical reactions.

Major Findings: The results of the kinetic studies of the conversion of prothrombin to thrombin that were reported last year are being prepared for publication.

Publications:

London, W. P. and Hearon, J. Z.: Estimation of the number of precursors in a sequence of first order reactions. Math. Biosci. 14: 281-295, 1972.

Hearon, J. Z. and London, W. P.: Path lengths and initial derivatives in arbitrary and Hessenberg compartmental systems. Math. Biosci. 14: 121-134, 1972.

Serial No. NIAMDD,ODIR, MRB 5.1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mathematical analysis of the dynamics of infectious diseases

Previous Serial Number: SAME

Principal Investigator: Wayne P. London

Cooperating Units: Professor James A. Yorke
Institute for Fluid Dynamics and Applied Mathematics
University of Maryland

Man Years:

Total:	.25
Professional:	.25
Others:	0

Project Description:

Objectives: To develop mathematical models of the dynamics of infectious diseases in large populations.

Major Findings: Recurrent epidemics of measles, chicken pox and mumps in cities are studied with a mathematical model of ordinary differential equations.

For each calendar month a mean contact rate (fraction of susceptibles contacted per day by an infective) is estimated from the monthly reported cases over a 30 to 35-year period. For each disease the mean monthly contact rate is 1.7 to 2 times higher in the winter months than in the summer months; the seasonal variation is attributed primarily to the gathering of children in school.

The mean monthly contact rates show systematic differences between the years with many cases and the years with few cases. In New York City the mean contact rates for chicken pox were different during the years 1931-1945 than during 1946-1960. The clustering of cases within social groups is proposed to account for these differences in the contact rates and for other empirical observations.

Computer simulations that use the seasonally varying contact rates reproduce the observed pattern of undamped recurrent epidemics: annual epidemics of chicken pox and mumps and biennial epidemics of measles. The two-year period of measles epidemics is the signature of an endemic infectious disease that would exhaust itself and become nonendemic if there were a minor increase in infectivity or a decrease in the length of the incubation period.

For populations in which most members are vaccinated, simulations show that the persistence of the biennial pattern of measles epidemics implies that the vaccine is not being used uniformly throughout the population.

The irregularity of epidemics of measles in cities of fewer than two million people can be explained by stochastic effects.

Epidemics of measles in distant metropolitan areas are highly correlated, but the reasons for correlation are not clear.

Serial No. NIAMDD, ODIR, MRB-6
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Theoretical effects of geometry on the electrophysiology of neurons

Previous Serial Number: NONE

Principal Investigator: Steven Goldstein

Man Years:

Total:	.8
Professional:	.7
Others:	.1

Project Description:

Objectives: To determine theoretically how the geometric properties of a neuron interacts with the electrical properties of the neural membrane. (In collaboration with Dr. W. Rall, NIAMDD-MRB)

Methods: Because of the difficulty in solving the existing mathematical models of the active neural membrane, it was necessary to use numerical methods. A particular model developed by Dr. W. Rall was solved numerically. Besides the relative ease of solution compared to the Hodgkin-Huxley equations, previous stability analysis of this model provided further insight into these solutions.

Results Obtained: The velocity of an action potential is strongly influenced by changes in the diameter of a model axon. Although velocity increases proportional to the square root of axonal diameter, it decreases as the rate of change of diameter increases. Thus, velocity is determined by the interaction of these two factors. An approximate relation between velocity and distance was obtained for arbitrary changes in diameter.

With regard to branching axons, it was found that the summation of the 3/2's power of branch diameters was as important in active propagation as in passive electrotonus. The value of this parameter determines the change in velocity in the region of the branch point. Conditions were observed where the direction of propagation could be reversed in the main branch upon reaching the branch point.

Further insights into the meaning of the terms safety factor and threshold were obtained. Also the relation between the shape and velocity of the action potential was defined more precisely.

The above results are being prepared for publication (with Dr. W. Rall).

Serial No. NIAMDD, ODIR, MRB-6.1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Automated recording of the medical history

Previous Serial Number: NONE

Principal Investigator: Steven Goldstein

Man Years:

Total:	.4
Professional:	.3
Others:	.1

Project Description:

Objectives: A major problem in automating the medical record is how to enter the medical history. The major deficiencies of present automated history systems are that either the physical-patient interview is eliminated or information is recorded at some time after the interview (causing inaccuracy). Another criticism is that much information is recorded which, in addition to being unnecessary, obscures the relevant information. The objective is to design a system that will eliminate these deficiencies.

Major Findings: Preliminary testing of a new system allowed the determination of detailed specifications for the design of an operational system. At present, the system has the following features. The computer presents a list of symptoms referable to the given organ system chosen by the physician. After a symptom is chosen, its characteristics may be modified (or not as the physician chooses), e.g., the model and time of onset, severity, frequency. This is accomplished by choosing from a second list, presented by the computer which depends on the symptom chosen. Information not found on a list may be typed into the computer. This information is to be recorded while the interview is in progress. The computer rearranges the information in chronological order before printing the history.

It was found that the system could be used to record the present illness during the interview without disrupting the flow of conversion.

Proposed Course: The preliminary analysis justifies further development of both the hardware and software components of the system. It is felt that this could be best accomplished in a clinical setting where interviews could be performed by multiple physicians and their suggestions incorporated into the system. A search for a group of interested physicians is being initiated.

Serial No. NIAMDD, ODIR, MRB-7
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Models for the development of the heterocyst spacing pattern in filamentous blue-green algae

Previous Serial Number: NONE

Principal Investigator: Richard Gordon

Other Investigators: Gabor T. Herman and Anthony E. Walsby

Cooperating Units: Department of Computer Science, SUNY/Buffalo; Department of Botany, Westfield College, University of London, England

Man Years:

Total:	0.25
Professional:	0.25
Others:	0

Project Description:

Objectives: Our goal is to understand how a simple multicellular pattern develops. Heterocysts in blue-green algae, which are single differentiated cells spaced more or less evenly along a blue-green algal filament one cell wide, have been chosen as a theoretically and experimentally accessible example of simple pattern formation.

Methods Employed:

Models are either worked out analytically or simulated using CELIA, a computer program developed at SUNY/Buffalo.

Major Findings:

1) A spacing pattern can be generated using:

a) mosaic models in which cells differentiate in a life cycle independent of their neighbors;

b) local interactive models, in which the behavior of a cell is influenced by its nearest neighbors;

c) wave models, in which the differentiation of cells depends on "chemical waves" going through them;

d) diffusion models, in which morphogen gradients are set up and differentiation is triggered by crossing threshold concentrations of morphogen within the cell.

2) An extensive computer-based bibliography on blue-green algae has been compiled.

Significance to Biomedical Research:

The development of spatial patterns of cells and tissues is a major unsolved problem. The heterocyst spacing pattern is amongst the simplest of such patterns; yet its elucidation is seen to require consideration of a number of alternative models.

Proposed Course:

The various models will be simulated to determine which will match available data on the distribution of distances between heterocysts. A manuscript in preparation will be submitted for publication.

Serial No. NIAMDD, ODIR, MRB-7.1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Three dimensional reconstruction from projections

Previous Serial Number: NONE

Principal Investigator: Richard Gordon

Other Investigators: Gabor T. Herman and Stuart Rowland, Marcello Barbieri, Harvey Pollard, Judith Prewitt and Douglas Reece, Julius Kane, Eduard Kellenberger

Cooperating Units: Department of Computer Science, SUNY/Buffalo; Institute of Histology and Embryology, University of Bologna, Italy; Reproduction Research Branch, NICHD; Data Management Branch, DCRT; Institute of Animal Resource Ecology, University of British Columbia, Vancouver, Canada; Department of Microbiology, Biozentrum, University of Basel, Switzerland.

Man Years:

Total:	0.25
Professional:	0.25
Others:	0

Project Description:

Objectives: 1) To develop the theory of reconstruction from finite numbers of projections. 2) To write computer programs for the data manipulations required for reconstructions in electron microscopy. 3) To reconstruct ribosomes and membrane proteins.

Methods Employed:

Reconstruction algorithms are developed and tested using a Fortran program (SNARK). SNARK has been modified to run as an interactive program on the DCRT PDP-10, with data in a form compatible with the program PEEP, which allows pictures to be seen and manipulated on the DEC340 display.

Data is collected from electron micrographs using a Joyce-Loebl microdensitometer.

Algebraic Reconstruction Techniques (ART) were initially devised by the principal investigator. The general problem is to ascertain the internal structure of an object from its projections (as in x-rays of a patient) taken from a selected number of angles.

Major Findings:

1) The unconstrained additive ART algorithm produces the solution of minimum variance of the equations obtained by taking the projections of the solution at each iteration.

2) An algorithm has been devised for locating potential and actual artifacts in reconstructions done from only a few projections.

3) Methods for deblurring ordinary tomograms and scintillation photographs using ART have been devised.

4) A comprehensive review of algorithms used in electron microscopy, radiology, radio astronomy and other fields has been completed.

Significance to Biomedical Research:

Three dimensional reconstruction techniques may soon have an importance in molecular biology comparable and similar to x-ray diffraction of macromolecules. The algorithms under study should also lead to improved performance of x-ray diagnostic devices, such as the EMI-scanner, as well as result in the design of more efficient three-dimensional radiological equipment.

Proposed Course:

1) The algorithms for locating artifacts and deblurring tomograms and scintillation photographs will be tested.

2) Computer programs for manipulating densitometer data for reconstructions will be made interactive on the PDP-10.

3) Reconstructions of ribosomes in microcrystals and proteins in the membranes of chromaffin granules in adrenergic cells will be attempted.

4) An algorithm will be sought for doing reconstructions when the angles are unknown. This case arises when only one high quality electron micrograph can be taken of a number of identical arbitrarily oriented structures. (Further exposures will degrade the particles.)

5) A conference on reconstruction methods is being planned in cooperation with the Optical Society of America.

6) A book on the intellectual history of reconstruction methods is planned.

Honors and Awards:

Dr. Gordon received an NSF travel grant to attend the Biophysics Congress in Moscow in August and visit Professor B. K. Vainshtein of the Academy of Crystallography. Drs. Herman, Barbieri and Gordon have received a NATO travel grant for collaboration on the structure of the ribosome.

Publications:

Gordon, R. and Herman, G. T.: Three dimensional reconstruction from projections: a review of algorithms. In Bourne, G. H., Danielli, J. F., and Jeon, K. W. (Eds.): International Review of Cytology, New York, Academic Press, Vol. 37, 1973 (In press).

Serial No. NIAMDD, ODIR, MRB-7.2
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Theories of function and development of the visual cortex

Previous Serial Number: NONE

Principal Investigator: Richard Gordon

Other Investigators: Helmut V. B. Hirsch, Michael Salcman

Cooperating Units: Center for Neurobiology of the Department of Biological Sciences, SUNY/Albany; Columbia College of Physicians and Surgeons, New York City

Man Years:

Total:	0.25
Professional:	0.25
Others:	0

Project Description:

Objectives:

1) To discover how the brain processes and stores visual images.

2) To discover the mechanism of the input-dependent postnatal development of the mammalian visual system.

Methods Employed:

A laboratory is being equipped at SUNY/Albany for the electrophysiological study of the function of single neurons in the cat's brain. Image processing by the cells will be studied using a computer controlled display and recording system. Chronic microelectrodes, which permit long-term developmental studies of single neurons, will be made in a laboratory at Columbia University.

To experimentally modify the functional properties of cells in the visual cortex, cats are limited from birth to seeing only chosen patterns. Each eye is shown a different pattern, so that two populations of cells with distinguishable properties are created. Each population can be independently activated and tested by stimulating only the appropriate eye.

Interactive computer simulations of developmental interactions are performed using the DCRT PDP-10. The reconstruction model is tested using the SNARK program.

Major Findings:

1) Many properties of vision can be explained by a model which assumes that the first step of processing in the visual cortex is to reconstruct an approximation of the retinal image using a scheme analogous to reconstruction from projections.

2) A basic feature of the brain's organization is that cells with similar functional properties are clustered together. We find that the development of such clusters or "columns" may be a consequence of nearest neighbor interactions which lead to a phase transition.

3) An experimental method for producing an ICON (Image Captured from an Optical Neuron) has been devised to explore the role of cells at any level in the visual system.

Significance to Biomedical Research:

Visual perception is an area of science in which we do not yet know how to state what we have to explain, let alone have a set of plausible theories to choose from. These explorations of the beginning of visual processing may lead to new insights into this problem. A correct theory of the function of the visual cortex is an essential step in the development of a visual prosthesis.

Proposed Course:

1) The computer simulation of the postnatal development of visual cortex cells in response to visual inputs and nearest neighbor interactions will be completed and used to design future experiments to test the model.

2) A manuscript on reconstruction models will be completed and submitted for publication.

3) Computer programs will be developed for data collection during experiments, construction of ICONs and determination of receptive field shapes. This will allow quantitative study of the responses of single neurons to very complex patterns at a level of sophistication previously unobtainable.

Serial No. NIAMDD, ODIR, MRB-7.3
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Computer simulation of the change of shape of the newt neural plate during early embryogenesis

Previous Serial Number: NONE

Principal Investigator: Richard Gordon

Other Investigators: Antone G. Jacobson

Cooperating Units: Zoology Department, University of Texas, Austin

Man Years:

Total:	0.25
Professional:	0.25
Others:	0

Project Description:

Objectives: The problem of morphogenesis is to explain how the development of the form of an organism is a consequence of the behavior of and interactions between its cells.

We chose as a model system the pattern of cell behavior which transforms the vertebrate neural plate (the embryonic brain) from a hemispherical to a keyhole shaped structure.

Methods Employed:

During the past three years we have developed computer programs capable of testing our understanding of the dynamics of change within the neural plate. Our investigations involved a continual interchange between experimental manipulations of embryos and computer simulations based on our hypotheses about the underlying morphogenetic mechanisms.

The California newt, Taricha torosa, was used because its neural plate is a monolayer sheet of cells, all visible at the surface of the developing egg. Experimental procedures included time-lapse movies, surgical removal of parts or the whole neural plate, and cutting of slits, whose gaping demonstrated directions of tension.

The computer program SHRINK (Simulated Hydrodynamic Reshaping into Neural Keyhole) was completed both on the SDS930 at the University of Texas and on the NIH PDP-10 in DCRT.

The program starts with a representation, using interactive graphics, of the neural plate as a disc divided into N hexagonally packed regions. (For N=100 each region would represent 100 cells.) Each region is assigned one of nine height programs $h_i(t)$, $i=1,\ldots,9$ (height perpendicular to the sheet as a function of time). Regions may be added, removed or have their height program changed, so that any surgical operation on embryos may be simulated.

Then the program shrinks the areas within the regions (volumes are assumed constant and $h_i(t)$ is generally monotonically increasing) repacking them using a Monte Carlo algorithm which retains the continuity of the sheet of cells they represent. The program displays the units as their positions change. Various display modes include: indices of the height programs; hexagons of proper radii; nearest neighbor connections; distortions of a D'Arcy Thompson grid. SHRINK also simulates the effect on the regions of a moving boundary representing the growing notochord. The resulting computer simulations can now be compared with the results of surgical operations performed on embryos.

Major Findings:

1) The change of shape of the newt neural plate is driven by and requires two forces: nonuniform shrinkage of the area of the cells, due to their increasing heights; and elongations of the notochord which is attached underneath.

2) There is extensive slippage and changing of cell-cell contacts in the neural plate.

3) A large part of the brain derives from the stage 13 blastopore region.

Significance to Biomedical Research:

The interplay of computer simulation and experiments is the beginning of a new quantitative approach to the development of vertebrate embryos in terms of the behavior of individual cells. It presents the possibility of investigating the relationship between form and cell behavior in teratology which may lead to a better understanding of the causes of some birth defects.

The discovery of slippage between cells maintaining a coherent sheet may be of importance in explaining organogenesis.

Proposed Course:

1) The above results are being prepared for publication.

2) The pattern of height programs has only been inferred from the match between the simulation and observations. We propose to reconstruct whole neural plates from serial sections, at different stages, representing the outer and inner surfaces by coordinates stored in the computer. These will be displayed three dimensionally and the heights measured directly using an interactive program.

3) An attempt will be made to derive and numerically integrate the partial differential equations governing the flow of the cell sheet by considering it as a problem in Lagrangian hydrodynamics.

4) Once the pattern of height programs is determined, we will begin to ask how it is formed.

ANNUAL REPORT OF CLINICAL INVESTIGATIONS, NIAMDD

July 1, 1972 to June 30, 1973

Robert S. Gordon, Jr., M.D., Clinical Director

During fiscal year 1973, NIAMDD clinical research has been strengthened by the transfer of two units from elsewhere within the NIH. Dr. Elizabeth Neufeld and her staff of five have transferred from Dr. Yale Topper's Section on Intermediary Metabolism in the Laboratory of Biochemistry and Metabolism, to reconstitute the Section on Human Biochemical Genetics within the Arthritis & Rheumatism Branch. This position had remained vacant for nearly four years, since the retirement of Dr. Jarvis Seegmiller in 1969. In addition, Dr. Paul Berk and his staff of five are in the process of transferring from the NCI Metabolism Branch to constitute a new Liver Unit within the Metabolic Diseases Branch of NIAMDD. Beginning July 1, 1973 Dr. Berk's patient admissions will be to the wards of NIAMDD, instead of to those of NCI. This change will require revision of bed assignments, and should result in an increased average percent occupancy.

Partially offsetting these gains, the Institute lost Dr. Scott Grundy, who moved from the Phoenix Clinical Research Section, where he had been head for two years, to a professorship at the University of California in San Diego. Because of the current limitation on positions, it has been decided that the Clinical Research Section will be reduced in strength, and supervised for an interim period by Dr. Peter Bennett who is also Chief of the Phoenix-based Epidemiology & Field Studies Unit.

For the period April 1, 1972 to March 31, 1973, the following data on inpatient admissions and outpatient visits apply: There were 556 inpatient admissions for a total of 13,390 hospital days, yielding an average length of stay of 24 days. The average bed occupancy remained low, 56%. There were altogether 2,635 outpatient visits to the many clinics operated by NIAMDD.

Data on the inpatient admissions have been sorted by computer and give rise to the following tables, which may be informative:

Admissions by Nursing Unit	
9-East	192
9-West	194
8-West	170
Total	556

Admissions by Branch		Assigned Beds	Admissions per Bed	Clinical Associates on Duty
ARB	162	15	10.8	7*
CEB	152	14	10.9	5
DHDB	97	11	8.8	3
MDB	64	10	6.4	3
PMB	51	7	7.3	2
CHB	30	9	3.3	2
Total	556	66	8.4	22

Admissions by Admitting Diagnosis	
Acromegaly	11
Cystic fibrosis	50
Dermatitis herpetiformis	22
Diabetes mellitus	17
Homocystinuria	12
Hypercalcemia/hyperparathyroidism	35
Normal volunteers	36
Obesity	16
Rheumatoid arthritis	41
Systemic lupus erythematosus	92
Sprue/malabsorption	23
Thyroid neoplasms	17
Others	184
Total	556

During FY 1973 there were 22 Clinical Associates on duty with NIAMDD. Other temporary professional and nonprofessional staff included five Visiting Scientists and 16 guest workers, representing seven foreign countries in addition to the United States.

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* One Clinical Associate appointed jointly by NIDR and NIAMDD assisted these 7 in patient care on 9-East.

ARTHRITIS AND RHEUMATISM BRANCH

Dr. Peter Goldman left the Branch during the year to become Professor of Pharmacology, Harvard University. The leadership of the Section of Human Biochemical Genetics has been assumed by Dr. Elizabeth Neufeld, currently transferring into the Branch.

Branch activities may be described under four major headings - studies of the pathogenesis of rheumatoid arthritis, studies of the nature and management of systemic lupus erythematosus, the use of affinity chromatography to remove albumin-bound substances from the blood, and studies of immunoglobulin function particularly on cell surfaces.

1. Rheumatoid Arthritis

It has been found that miniature swine of the Piney Woods strain fail to develop a chronic, destructive, microbiologically-sterile synovitis such as we have reported in Yorkshire swine. The finding is disappointing in excluding the smaller animals from laboratory studies but the greatly contrasting susceptibility of the two breeds would appear to be another feature shared with the human disease, rheumatoid arthritis (Decker and Aptekar). The study of prophylactic synovectomy, carried out upon a randomly selected joint from a pair of equally diseased joints, continues. The early results clearly favor the operated knee but the advantage appears to decrease to the vanishing point about 3 years postoperatively (Decker, Aptekar, Peterson (not NIH), and Fried (CC:Rehab)).

Incorporation of tritiated uridine into RNA as a measure of human lymphocyte response to antigens is proving useful because so many replicates can be performed on a single blood sample (Seaman).

2. Systemic Lupus Erythematosus

The Branch is regularly caring for approximately 40 patients with SLE and most of these have taken part in therapeutic trials (see below). The availability of this carefully followed group has permitted a number of studies to move forward. The nests of tubuloreticular structures (TRS), originally identified by electron microscopic examination of endothelial cells, are now regularly seen in lymphocyte sections made from patient buffy coats. Despite the similarity of the structures to those found in white blood cell long term cultures from patients with infectious mononucleosis, no correlations between the structures and EB virus antibody were found in our patients. Sequential studies of lymphocyte TRS suggest that their disappearance from the blood is associated with decreased disease activity. Lymphocytes positive for TRS have been found in the cord blood of infants born to SLE mothers, a finding which suggests to us that the change is induced in the cells of the healthy infant by a maternal antibody (Decker, Grimley (NCI), Klippel, and Evans (not NIH)).

Prospective neuropsychiatric studies in patients with SLE do not support the view that psychiatric crises are related to reduction of certain components of hemolytic complement in the cerebrospinal fluid (Hadler, Frank (I), Gerwin (I), Whitaker (N), Baker (M), Donnelly (M), and Decker). Studies of delayed hypersensitivity (both pre-existing and induced) and humoral immunity in our patient group show that patients with SLE have markedly impaired reactivity. There is some evidence of improvement in this picture as the patient improves on "immunosuppressive drugs" (Decker, Steinberg, Aptekar, and Waldmann (C)). Extended assessment of urine fibrin degradation products has shown that these products of inflammation are not good predictors of the activity of lupus nephritis as judged by the more usual, routine modes for such determinations (Decker, Steinberg, Gralnick (CC), Marchesi (CC), and Aptekar).

A therapeutic trial of the management of diffuse lupus nephritis involving 38 patients has been completed. The patients were each closely observed over a 10 week inhospital course of blindfold, randomly assigned 1) continuation of steroid therapy only, 2) previous medications plus cyclophosphamide, or 3) previous medications plus azathioprine. The results show that, in the short term, cyclophosphamide is better than azathioprine, and azathioprine is better than placebo. In the longer term, infections and other "toxicity" problems in the treated group greatly reduce the advantage to the cytotoxic agents. Detailed reports are in preparation (Decker, Steinberg, and Aptekar). Certain SLE patients, too sick to meet the admission criteria of the above study, have been randomly assigned to receive intermittent intravenous cyclophosphamide or saline. Five patients have been admitted; the code has not been broken (Plotz, Decker, and Steinberg).

In the course of using large doses of aspirin in the treatment of SLE, several patients have shown sharp increases in serum transaminase levels. We have now seen three cases where this situation became a crucial consideration in the diagnosis of chronic active hepatitis or "lupoid hepatitis." The problem is under systematic study; aspirin may be a cause for chronic active hepatitis, a relationship which has only recently been identified for a common laxative, oxyphenisatin (Seaman and Plotz).

The pathogenesis of the disease continues under study using the spontaneous illness which develops in the New Zealand Black/New Zealand White mouse hybrid. Here the emphasis has been on thymic suppressor cells which act over the full lifetime of regular species but appear to disappear very early in life (one to two months) in the NZB/W hybrid. The deficiency of this suppressor effect has been detected both in humoral immunity (using a thymus-independent antigen) and in delayed hypersensitivity (using the graft-versus-host system). The survival of the hybrids can be prolonged by the repeated grafting of syngeneic young thymus cells (Steinberg, Parker, Gelfand (I), Scher (not NIH), Gazdar (C), Powell (not NIH), Chused (D) and Hardin (D)).

In therapeutic studies it was found that no known agent (including adrenal corticosteroids, azathioprine, and cyclophosphamide, either singly or in combination) favorably modified the disease once renal damage, as detected by proteinuria, had supervened; somewhat comparable findings in the human disease have been cited by numerous investigators in the past (Steinberg, Gelfand (I), Hardin (D) and Schur (not NIH).

3. Eliminating Albumin-Bound Products

A new area of work has been opened by the finding that affinity-chromatography can be applied to the removal from whole blood of such albumin-bound products as bilirubin, thyroxine and salicylates. Human albumin was conjugated to agarose beads. Rat whole blood containing large amounts of bilirubin can be passed over such a column without apparent damage to the formed elements; the effluent blood is virtually free of bilirubin and the pigment can be eluted from the column and quantitated (Plotz, Gordon, Scharaschmidt (C), Berk (C), Vergalla (C) and Waggoner (C)).

4. Immunoglobulin Function

Exploration of immunoglobulin binding sites has continued using induced mouse myeloma proteins which bind phosphoryl-choline and can be probed with the affinity labeling reagent paranitrophenyl phosphoryl choline. It was notable that 2 independently developed tumors, TEPC 15 and HOPC 8, gave virtually identical binding data with the probe labeling two identified light chain tyrosine residues in each. Two proteins were found which labeled primarily on the heavy chains and one of these, MOPC 603, has a crystalizable Fab fragment. It is hoped that x-ray diffraction analysis will, for the first time, be able to specify directly the relationship between label and binding site (Metzger, Chesebro, Hadler and Carson).

In a series of studies, using hybrid antibody ferritin labeling for electron microscopy and fluorescein labeling for ultraviolet microscopy, it has been conclusively shown that histamine release from basophils is not dependent upon the demonstrable redistribution and capping of the cell surface Immunoglobulin-E (Metzger, Becker, Grimley (C), Ishizaka (not NIH) and Ishizaka (not NIH)).

Mast cells also release vasoactive amines when reaginic IgE on their surfaces reacts with its specific antigen. The IgE binding site and its mode of action as a cell surface transducer is under study. Cells from two transferable mouse mastocytomas have, in an absorption procedure, been shown to deplete mouse serum of reaginic IgE. To date, the demonstration that the immunoglobulin is, in fact, on the cells has proven difficult because it is so readily washed off. Nevertheless, it is believed that the results of this work will bear on the important problem of how antigen-antibody reactions initiate specific cellular responses. (Metzger, Kulczycki and Isersky-Carter).

Serial No. NIAMDD-ARB- 1c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Role of Infection in Rheumatoid Disease

Previous Serial Number: NIAMD-ARB-1c

Principal Investigator: Dr. John L. Decker

Other Investigators: Robert G. Aptekar, M.D.

Cooperating Units: None

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

The overwhelming likelihood is that rheumatoid arthritis is due to an infection. There is a long history of failure in trying to isolate the responsible microbial agent. We therefore believe it important to study the biology of the host-parasite relationship using a known microbial agent to produce an animal disease similar to rheumatoid arthritis. To this purpose we have been assessing the events in the evolution of chronic Mycoplasma hyorhinis arthritis of swine.

Methods Employed:

The polyarthritis which develops following intraperitoneal injection of Mycoplasma hyorhinis has been studied over an 18 month period by: repeated clinical evaluation; serial determination of serum proteins and of serum antibody activity to M. hyorhinis; serial blood cultures; sequential autopsies with material for culture, tissue culture, immunofluorescent studies, and histopathology; sequential synovial fluids for antibody activity.

Major Findings:

In contrast to Yorkshire swine, it has been found that miniature swine (of the Pney Woods strain) develop no chronic arthritis. Histological specimens from control and infected animals extending to 340 days post-inoculation reveal a transient acute arthritis but no permanent damage and no chronic inflammation. Organisms were not recovered after the 101st day and appear to have been eradiacted by the host.

The marked contrast between the two breeds, Piney Woods miniatures as compared to Yorkshire, is reminiscent of discussions of genetic susceptibility of humans to rheumatoid arthritis. The divergent mechanisms by which the animals react to produce these results are of great importance and continue under study.

Proposed Course:

Two manuscripts are now in preparation. No more field work is now planned.

Significance to Bio-Medical Research and the Program of the Institute:

The two strains, one with and the other without chronic arthritis, tend to reinforce the potential relationship to rheumatoid arthritis.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-2c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Retrospective Evaluation of Knee Synovectomy in Rheumatoid Arthritis

Previous Serial Number: ARB-3c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Robert G. Aptekar, Dr. Leonard T. Peterson, Orthopedic Consultant, George Washington University School of Medicine and Dr. David Fried (CC:Rehab)

Cooperating Units: George Washington University School of Medicine, Washington, D. C. and Clinical Center, Rehabilitation Department.

Man Years:

Total:	1-1/4
Professional:	1/2
Other:	3/4

Project Description:

Objectives:

To discover whether or not prophylactic synovectomy, as defined below, will alter the functional or structural prognosis in rheumatoid arthritis.

Methods Employed:

Patients with rheumatoid arthritis who have had at least six months of active inflammation in both of a pair of symmetrical joints, neither of which show radiological changes other than osteoporosis, have been admitted to the study. The disease must be sufficiently symmetrical to have all parties -- the investigator, the surgeon and the patient -- agree to have either one of the pair operated. The side to be done is selected by lot.

The patients are then seen at intervals of six months, one year, and two years. On these visits they undergo standard evaluation for function and radiological examination. Extended follow-up for as long as five years has been completed.

Major Findings:

Thus far it is possible to say that, at 18 months followup, the operated knee was still preferred clinically. The radiological changes have not been impressive in either knee. Twelve patients have been studied to date. It appears that the most important determinant of successful surgery has been the general state of the arthritis.

Significance to Bio-Medical Research and the Program of the Institute:

There has been essentially no controlled work on this important therapeutic modaility which is being widely urged on the basis of inadequate data. There is reason to believe that, almost alone among treatment methods, it will prevent joint damage. This needs to be established.

Proposed Course:

Preliminary analysis of the initial group is underway.

It is proposed to continue to admit suitably selected patients to the study.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-3c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Early RNA synthesis by immune lymphocytes in response to antigen.

Previous Serial Number: None

Principal Investigator: Dr. William Seaman

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/2
Other	0

Project Description:

Objectives:

Present methods for detection of delayed hypersensitivity (DH) in human lymphocytes are tedious and slow. Some studies have shown very early increases in protein synthesis in response to antigens. The object of these studies is to see whether the incorporation of uridine into RNA will provide a more rapid and sensitive assay for DH in human lymphocytes.

There is no satisfactory system on studying DH in mice at present. A second objective is to develop such a system for mouse lymphocytes.

Methods Employed:

Lymphocytes are separated from peripheral blood (human) or spleen (mouse) and cultured for up to twenty-four hours with or without antigen and then given a brief pulse of ^{3}H-uridine. The incorporation of counts into acid precipitable RNA is measured.

Major findings:

There is an early antigen-induced RNA stimulation in sensitized human lympocytes. Whether this will be adaptable as a practical test is not clear yet.

Significance to Bio-Medical Research and the Program of the Institute:

A rapid easy test for human DH would be of great value in studying the role of abnormal immunity in the development of auto-immunity in connective tissue diseases. In mice, such a test could allow studies of DH in an animal widely exploited for immunoglobulin research but presently almost unavailable for studies of cellular immunity.

Proposed Course:

Continued investigation of the specificity of the antigen-induced changes.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-4c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Tubuloreticular Structures of Circulating Lymphocytes in Systemic Lupus Erythematosus

Previous Serial Number: ARB-7c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Philip M. Grimley (NCI), John H. Klippel, M.D., Dr. Alfred S. Evans (Yale Univ. School of Medicine).

Cooperating Units: National Cancer Institute, Laboratory of Pathology and Department of Epidemiology and Public Health, Yale University School of Medicine.

Man Years:

Total:	1-3/4
Professional:	1-1/4
Other:	1/2

Project Description:

Objectives:

Nests of interwoven tubulorecticular structures (TRS) have been recognized by electron microscopy in the cytoplasm of the endothelial cells of kidney and skin from patients with systemic lupus erythematosus for several years now. TRS, also identified in endothelial cells from cases with Sjogren's syndrome, polymyositis and scleroderma, are considered by some to be paramyxovirus particles but others, finding them associated with a number of experimental viral infections, interpret them as nonspecific cellular response to viral infection.

Upon examining a skin biopsy specimen from one of our patients with SLE, TRS were seen not only in the endothelim as expected but also within the cytoplasm of a circulating mononuclear cell, probably a lymphocyte.

The objective of study was to assess the prevalence of TRS in buffy coat lymphocytes from patients with connective tissue disease. This objective is consonant with the larger objective of understanding the pathogenesis of SLE and related diseases.

Methods Employed:

We have continued to prepare and examine lymphocyte specimens from our patients with SLE approximately every 6 months and the results are being compared to determinations of the prevalence in them of thymus derived (T) or bone marrow derived (B) lymphocytes.

Dr. Evans has studied sera of 75 of our subjects measuring the EBV antibody titers by an indirect immunofluorescent technique using the EB3 cell line.

Major Findings:

1. TRS containing lymphocytes have been identified in the cord blood of two normal infants born to mothers with SLE under our care. The number of TRS ⊕ lymphocytes in the cord blood was such that they could not have been maternal lymphocytes unless a totally unknown form of placental concentration is occurring. We believe the finding to be evidence of an effect of maternal antibodies on infant cells; maternal antibodies have frequently been found in cord blood.

2. There was no correlation between the presence of lymphocyte TRS and antibody titers to EBV in the same patient's sera. In our patients the EBV antibody appeared to correlate with immunoglobulin levels of both G and M classes.

3. In our sequential studies, 18 patients with SLE have been studied on more than one occasion over an average span of 8 months. In 14 all studies have been concordant; 9 were always positive and 5 were always negative. In four patients there has been a change; all four were initially positive and became negative. In all four the change was associated with substantial improvement in the patient and a notable decrease in disease activity.

Significance to Bio-Medical Research and the Program of the Institute:

While others have suggested that these structures represent some form of myxovirus nucleocapsid, our evidence suggests that they are secondary to circulating antibody.

Proposed Course:

We propose to carry out a variety of incubations or normal lymphocytes with sera from patients with SLE in an effort to induce the changes.

Honors and Awards: None

Publications:

Grimley PM, Decker JL, Michelitch HM, Frantz MM: Abnormal structures in circulating lymphocytes from patients with systemic lupus erythematosus and releated diseases. Arthritis Rheum (In press).

Serial No. NIAMDD-ARB-5c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the pathophysiology of nervous system involvement in systemic lupus erythematosus (SLE)

Previous Serial Number: ARB-9c

Principal Investigator: Dr. Nortin M. Hadler

Other Investigators: Dr. Michael Frank (I:LCI), Dr. Robert Gerwin (I:LCI), Dr. John Whitaker (N:IR), Dr. Max Baker (M:DCBR), Dr. Edward Donnelly (M:AP) and Dr. John L. Decker

Cooperating Units: National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigations; National Institute of Neurological Diseases and Stroke, Intramural Research; National Institute of Mental Health, Division of Clinical and Behavioral Research.

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Objectives:

To attempt to relate the neuropsychiatric crises of hospitalized patients with SLE to the hemolytic titer of the fourth component of complement (C'4) in the cerebrospinal fluid (CSF).

Methods Employed:

C'4 is titered in fresh CSF (and concurrently sampled serum) samples, utilizaing C'4-deficient guinea pig sera in a hemolytic molecular assay. Lumbar punctures are performed at the start and, when possible, at termination of hospitalization and at times during the course when a change in neuropsychiatric status is noted. Patients are evaluated initially and serially by a neurologist and psychiatrist, and subjected to a thorough battery of psychological testing initially and after 10 weeks of in hospital evaluation.

Major Findings:

In contrast to the relationship of the C'4 level of the CSF to change in neurological status, no such has been detected in relationship to extensive psychiatric observations carried out prospectively. Although some evidence of adrenal corticosteroid induced psychiatric pathology was detected, our studies reinforce the view that most of the psychopathology is related to the disease activity of SLE itself.

Significance to Bio-Medical Research and the Program of the Institute:

This is the first systematic longitudinal study of neuro-psychiatric involvement in SLE.

Proposed Course:

No further work is planned on this project. Two reports dealing particularly with the psychiatric manifestations of systemic lupus erythematosus are in preparation.

Honors and Awards: None

Publications:

Hadler NM, Gerwin RD, Whitaker JN, Baker M, Decker JL: The fourth component of complement in the cerebrospinal fluid in systemic lupus erythematosus. Arthritis Rheum (in press).

Serial No. NIAMDD-ARB-6c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cellular and humoral immunity in systemic lupus erythematosus (SLE) undergoing randomized cytotoxic therapy.

Previous Serial Number: None

Principal Investigator: John L. Decker, M.D.

Other Investigators: Alfred D. Steinberg, M.D., Robert G. Aptekar, M.D., and Thomas A. Waldmann, M.D., (C:MET)

Cooperating Units: National Cancer Institute, Metabolism Branch.

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

The literature provides a variety of statements as to the capacity of patients with SLE to respond to antigens which stimulate humoral and delayed hypersensitivity responses. There is evidence that these are altered by the disease itself (especially when active) but such studies are always perturbed by the use or the non-use of adrenal corticosteroid therapy which is essential to the patients' well-being. Virtually nothing is known of the effect, on this background, of the cytotoxic drugs azathioprine and cyclophosphamide. We designed a study to answer the question do azathioprine or cyclophosphamide, given in clinically useful doses, actually produce immunosuppression in SLE as contrasted to the effects of placebo?

Methods Employed:

Twenty-five patients were evaluated by skin tests to assess delayed hypersensitivity and by circulating antibody titers to assess humoral immunity before and during a controlled trial of the use of prednisone alone or in combination with cyclophosphamide or azathioprine. Standard skin test preparations of PPD, mumps, SK/SD, candida, trochophyton and mixed respiratory vaccine were used. Purified polysaccharide antigens of pneumococcus Type I (Pn I) (given before study), Type II (Pn II), the Vi antigen of E.coli, KLH and BRU (all given during study) were used to stimulate circulating immunity.

Major Findings:

Skin test results were not appreciably different in the 3 treatment groups. None of 7 patients on prednisone could be sensitized to DNCB but 3 of 8 were positive on azathioprine and 1 of 3 on cyclophosphamide.

Mean antibody levels to all antigens except Vi were the same in all 3 groups and were lower than non-SLE controls. The response to Vi was significantly depressed only in patients on azathioprine.

Significance to Bio-Medical Research and the Program of the Institute:

Drug therapy apparently facilitates the cellular reaction to DNCB. This may be important in improving the prognosis of patients with SLE in terms of decreasing the likelihood of infection. Impairment of cellular immunity appeared to be greater than that of humoral immunity.

Proposed Course:

This study has been completed and a manuscript is in preparation.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-7c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Systemic Lupus Erythematosus Coagulation Study

Previous Serial Number: NIAMD-ARB-10c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Alfred D. Steinberg, Dr. Harvey Gralnick (CC-CP), Dr. Sally Marchesi (CC-CH), and Dr. Robert Aptekar

Cooperating Units: Clinical Center, Clinical Pathology Department

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

To study abnormalities of coagulation in patients with systemic lupus erythematosus (SLE), and to correlate these abnormalities with disease activity.

Methods Employed:

Patients with SLE who are admitted to the Clinical Center and selected patients with SLE seen in the out-patient department are studied.

Evaluation of Clinical status is done independently by Drs. Decker and Steinberg and the results recorded on separate evaluation sheets. On the same day, the patient's blood is studied for coagulation factors, fibrin split products, and other hematological parameters. A 24-hour urine is also studied for fibrin split products. The coagulation study by clinical hematology is performed without knowledge of the clinical evaluation.

Major Findings:

Patients with active nephritis had elevated fibrin degradation products (FDP) excretions ranging from 1 to 50 mg. per 24 hours and the highest excretions were generally found in those with most active disease.

However, sequential assessment of 19 patients showed that FDP excretion fluctuated widely and without apparent relationship to disease. The measure was not correlated with creatinine clearance, urinary protein, serum anti-DNA antibodies, nor serum complement values.

Significance to Bio-Medical Research and the Program of the Institute:

We concluded that the measure was not of clinical merit at our present level of comprehension.

Proposed Course:

A final report is in preparation.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-8c
1. Arthritis and Rheumatism
2. Connective Tissue Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Controlled study of cyclophosphamide and azathioprine in systemic lupus erythematosus with nephritis

Previous Serial Number: 12c

Principal Investigators: Dr. John L. Decker and Dr. Alfred D. Steinberg

Other Invesitgators: Dr. Robert Aptekar

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

An earlier study (Serial No. ARB-6c, 1971) has demonstrated that cyclophosphamide has a substantial effect on systemic lupus erythematosus (SLE). Reductions of anti-DNA antibodies and increases in serum complement values were more impressive than was amelioration of renal disease. Azathioprine is being widely used by others in SLE. Others have shown that cyclophosphamide has substantially more effect on NZB/W mouse renal disease, a model of lupus nephritis, than does azathioprine.

In view of the significant toxicity of both drugs, it is important to compare them directly within a single trial. The objective are to assess the therapeutic efficacy/toxicity of azathioprine and cyclophosphamide in comparison to each other and to placebo in SLE nephritis.

Methods Employed:

Patients meeting predefined criteria for diagnosis and activity of nephritis will be admitted to the 12 week trial. In the two week baseline control period, the patients will be kept on the anti-inflammatory regimen (usually prednisone and aspirin) needed to control extra-renal manifestations. These will be continued into the ten week study period with as little change as possible and with the addition of randomly assigned cyclophosphamide, azathioprine or placebo. Dosage of each drug will be 3-4 mg/kg/day unless leukopenia (less than 3,000 WBC/mm^3) requires reduction below that level.

A large number of serological, chemical, and urine changes will be followed in the course. Immunoglobulin levels, anti-DNA antibodies, serum complement (by hemolytic and by precipitin assay), urinary fibrin split products, and the response to a number of antigens (for delayed hypersensitivity and circulating antibodies) are among those parameters.

Major Findings:

Thirty-eight patients (33 females and 5 males) with systemic lupus erythematosus, diffuse glomerulonephritis on biopsy and clinically active renal disease were hospitalized for a ten week double blind therapeutic study. Patients were given oral azathioprine (AZ), cyclophosphmmide (CY), or placebo (PL) from a table of random numbers starting at a dose of 3.0 mg/kg/day (mkd). The drug dose was adjusted on the basis of white cell count; it could be raised to 4.0 mkd or lowered. Steroids up to 0.5 mkd prednisone were allowed for control of extra renal symptoms; average dose in mg/d was 19.8 for CY patients, 21.3 for AZ and 25.2 for PL. The results are summarized below:

				Number Improved minus Number Worsened				
Drug	No.	Ccr	Proteinuria	Sediment	C'3	Anti-DNA	Non-Renal	Total
CY	10	3	3	8	5	4	0	23/60
AZ	13	1	1	0	1	7	-1	9/78
PL	15	-1	-5	-1	0	2	-6	-11/90

CY (average 1.8 mkd) was significantly better than both AZ and PL for all 6 measures and for the 3 renal measures. AZ (average 2.4 mkd) was significantly better than PL for all 6 measures but not for the 3 renal measures. IgM fell in all patients receiving CY (mean -34%) while there was no change in IgG. Both IgM (mean -20%) and IgG (mean -23%) fell during AZ therapy. Quantitative determination of antibodies to DNA showed reduction in all groups averaging AZ - 48%, CY - 42%, PL - 18%. Circulating lymphocytes fell 81% in CY patients but only 16% in AZ patients. The lymphocyte count fell below 400/mm^3 at least once in all CY patients but in only 5/13 AZ and 3/15 PL patients. Granulocytes fell below 500/mm^3 in only 2 patients, both on AZ.

Major complications (death, dialysis, infection or other manifestations requiring a major change in drug dosage) to 2/1/73 were: AZ 6 in 218 months (1/3.0 patient-years), CY 3 in 195 months (1/6.4 patient-years), PL 5 in 151 months (1/2.5 patient-years). The percent of patients alive and not requiring dialysis as of 2/1/73 was PL 75%, CY 88%, AZ 89%. We conclude that in 10 weeks CY is superior to both AZ and PL, and AZ is superior to PL. More long term data are necessary before clinical recommendations are possible.

Significance to Bio-Medical Research and the Program of the Institute:

If differences in the response of the disease and some of the immunological processes can be detected between the alkylating agent and the purine analogue, it will be possible to draw conclusions in reference to the cell populations involved in systemic lupus erythematosus and thus extend our understanding of the disease process itself. It is expected that the results will permit us to select between the two in ordinary clinical management.

Proposed Course:

A long-term followup of these patients will take place by one-week admissions every 6 months.

Honors and Awards: None

Publications:

Steinberg AD, Decker JL, Aptekar RG: Completed ten week trial of cyclophosphamide or azathioprine or placebo in lupus nephritis. Arthritis Rheum (Abst) 16: June-July, 1973.

Serial No. NIAMDD-ARB -9c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Therapy of SLE: A controlled trial comparing high dose intravenous cyclophosphamide with placebo.

Previous Serial Number: 14C

Principal Investigator: Dr. Paul H. Plotz

Other Investigators: Dr. John L. Decker
Dr. Alfred D. Steinberg

Cooperating Units: None

Man Years:

Total:	3/4
Professional:	1/4
Other:	1/2

Project Description:

Objectives:

At present, no satisfactory therapy exists for severe manifestation of SLE except for renal disease. Even renal disease does not respond well to conventional therapy when the creatinine clearance is markedly reduced. In this study we will test the efficacy of high dose intravenous therapy with cyclophosphamide for the control of severe SLE unresponsive to a trial of high dose of prednisone (up to 2 mg per kg for 3 weeks).

Methods Employed:

Patients with 1) severe renal disease or rapid deterioration of renal function 2) sensitivity to azathioprine precluding the CAP protocol 3) severe extra-renal lupus (seizures, hemorrhage, psychosis, hemolytic anemia, myocarditis, or pericarditis uncontrolled by high-dose prednisone) will be randomly assigned to receive intravenous cyclophosphamide or placebo for 3 initial injections over 6 weeks and then at 3 month intervals. Renal and non-renal patients will be separately randomized. The study will be double-blind: neither physicians nor patients will know which therapy is being administered. The end point of the study will be death or the need for renal dialysis except that patients well for 1 year may have therapy stopped. The potential hazards and benefits will be explained in full to all patients or their families.

Major Findings:

Five patients have been admitted to the study so far - one in the non-renal group and two in the renal group. No findings of significance to date.

Significance to Bio-Medical Research and the Program of the Institute:

The therapy of SLE has been under study here for several years. The present study is an attempt to extend the therapy for patients unresponsive to conventional therapy.

Proposed Course:

We plan to enter 20 to 30 patients into the study over the next 3-5 years.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-10c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Antibodies to Double Stranded DNA and RNA in Spontaneous and Drug-Induced Systemic Lupus Erythematosus and other Diseases

Previous Serial Number: 8C

Principal Investigator: Dr. Alfred Steinberg

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1/4
Other: 3/4

Project Description:

Objectives:

Study the specificity of anti-DNA and anti-RNA antibodies in human and murine lupus and their frequency in other diseases.

Methods Employed:

Farr ammonium sulfate precipitation assay, using single or double stranded C^{14}-KB cell DNA, or C^{14} polyinosinic·polycytidylic acid as antigen. Standard precipitin reaction using unlabelled nucleic acids.

Major Findings:

1. Antibodies to double stranded DNA and RNA are found respectively in 75% and 45% of human lupus patients, and rarely in other human autoimmune disorders, including drug-induced lupus.

2. Inhibition studies demonstrate that these are largely distinct antibody populations that may be directed against viral nucleic acids. Antibodies to RNA have specificity for several double-stranded RNAs investigated. Such antibodies arising spontaneously in B/W mice seem to have greater affinity for RNA than similar antibodies appearing in human lupus.

3. Antibodies to DNA and RNA were quantitated and found to represent a significant percentage of all antibody in certain instances.

4. Early antibody responses to nucleic antigen are IgM, late IgG.

5. Regulatory control of the antibody response to a nucleic acid antigen is derranged in murine SLE.

Significance to Bio-Medical Research and the Program of the Institute:

The presence of antibodies to double-stranded DNA and RNA strengthens the possibility of viral antigens in human lupus. Derranged regulatory function may help understand the pathogenesis of these autoantibodies.

Proposed Course:

Continued clinical study of anti-DNA and anti-RNA antibodies in SLE and other diseases. Study of the specificity of antibodies to nucleic acids in drug-induced SLE. In view of the relationship between Australia antigen and connective tissue disease and between SLE and chronic hepatitis, antibodies to nucleic acids in hepatitis (before, during and after) will be studied.

Honors and Awards: None

Publications:

1. Schur PH, Stollar BD, Steinberg AD, et al: Incidence of antibodies to double-stranded RNA in systemic lupus erythematosus and related diseases. Arthritis Rheum 14:342-347, 1971.

2. Parker LM and Steinberg AD: The antibody response to polyinosinic·polycytidylic acid. J Immunol 110:742, 1973.

3. Scher I, Frantz M, Steinberg AD: The genetics of the immune response to a synthetic double stranded RNA in a mutant CBA mouse strain. J Immunol, in press.

4. Schused TM, Steinberg AD, Parker LM: Enhanced antibody response of mice to polyinosinic·polycytidylic acid by anti-thymocyte serum and its age-dependent loss in NZB/W mice. J Immunol, in press.

Serial No. NIAMDD-ARB-11c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The hepatic toxicity of aspirin

Previous Serial Number: None

Prinicipal Investigator: Dr. William Seaman

Other Investigators: Dr. Paul Plotz

Cooperating Units: None

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

Recent observations have suggested that certain patients with connective tissue diseases develop abnormal liver function tests when they take large doses of aspirin. We too have observed this in patients with systemic lupus erythematosus (SLE) but not with rheumatoid arthritis (RA). The abnormalities may be so profound as to suggest the diagnosis of chronic active hepatitis. We wish to define the population at risk more exactly and determine what factors contribute to toxicity.

Methods Employed:

Patients with connective tissue disease are admitted to the Clinical Center. They may or may not have an immediate indication for the institution of aspirin therapy. Aspirin is administered in increasing doses to achieve serum salicylate levels of 30 mg% for two weeks while liver function is tested. At the end of that period, the drug is continued at the clinically indicated level.

Major Findings:

So far none of five adults with RA, one adult with juvenile JRA, and three of six adults with SLE have shown abnormalities of liver function while taking aspirin.

Significance to Bio-Medical Research and the Program of the Institute:

The safe use of aspirin in patients with connective tissue disease has been taken for granted. We believe that in patients with SLE there is a significant risk of inducing a hepatitis so severe as to lead to a misdiagnosis of chronic hepatitis. Awareness of this problem may diminish the risk of inducing hepatitis in patients with SLE.

Proposed Course:

We plan to admit further patients to this study as it now stands. When a group at risk is better defined, we expect to study aspirin binding and metabolism in relation to hepatortoxicity.

Honors and awards: None

Publications: None

Serial No. NIAMDD-ARB- 12c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Pathogenesis of Autoimmunity in New Zealand Mice

Previous Serial Number: 5c

Principal Investigator: Dr. Alfred D. Steinberg

Other Investigators: Dr. Leroy Parker, Dr. Michael Gelfand (NIAID), Dr. Irwin Scher (NNMC), Dr. Adi Gazdar (C-VLL), Dr. Deborah Powell (Georgetown University Hospital), Dr. Thomas Chused and Dr. John Hardin (D-OMS).

Cooperating Units: NIAID, National Navy Medical Center, NCI-VLL, Georgetown University Hospital and D-OMS Branch.

Man Years:

Total:	2
Professional:	1-1/4
Other:	3/4

Project Description:

Objectives:

New Zealand mice spontaneously develop an autoimmune disease resembling human systemic lupus erythematosus. Early in life they become relatively resistant to tolerance induction, later they spontaneously develop antibodies to RNA and DNA and finally they develop immune complex glomerulonephritis from which they die. Both humoral and cellular immune defects have been reported and are under active investigation.

Cellular cooperation between thymus derived (T) cells and bone marrow or bursa derived (B) cells is a recently described feature of humoral immunity. Two distinct T cell subpopulations cooperate in cell mediated immunity. The existence of suppressor T cells has been suggested by the increased antibody response to a thymic independent antigen given with antithymocyte serum (ATS). We have been investigating such suppressor cell function in New Zealand mice in the hope of understanding the pathogenesis of thei autoimmune disease.

Methods Employed:

1. Assay for antibodies to RNA and DNA using ^{14}C-labelled nucleic acids as ligands and the ammonium sulfate precipitation assay. Assay of antibody forming cells to sheep erytherocytes and polysaccharide by local hemolysis in gel.

2. Immunization of animals with a variety of antigens with and without protein carriers or adjuvants.

3. Transfer of spleen, bone marrow and thymus cells into recipients to evaluate the functional properties of the different cell types.

4. Use of standard skin grafting using allogenic skin to evaluate cellular immune functions in New Zealand mice, and transfer of serum and cell population between young and old New Zealand mice to study the effect in New Zealand mice and other mice.

5. Study of graft versus host disease inducing properties of NZB/W lymphoid cells using the Simonsen spleen weight assay and new born C3H/mice as recipients.

6. Separation of lymphoid cells functionally (corticosteroid, ATS, anti Θ, anti kappa) and physically (sedimentation).

Major Findings:

1. The double stranded RNA, rI·rC, induces an antibody response without carrier protein or adjuvant. This response has been found not to require much thymic helper function. Simultaneous administration of rI·rC and rabbit anti-mouse thymocyte serum (ATS) led to an increased response in control strain of mice suggesting that suppressor T cells were being eliminated by the ATS. Young NZB/W mice also showed enhancement; however, 5 and 6 month old NZB/W unlike control mice of the same age showed no enhancement with ATS. This suggested an age-dependent loss of suppressor cells to a nucleic acid antigen in NZB/W mice.

2. Female NZB mice were found to have a progressive increase in antibody response to the thymic independent antigen, type III pneumococcal polysaccharide (SSSIII). This suggested a more general loss of suppressor function. Thymic cells from one month old NZB mice significantly reduced the excessive antibody response to SSSIII of 10 month old NZB mice. TWo month old thymic cells were much less effective indicating a reduciton in thymic suppressor cells early in life.

3. A similar age-dependent loss of suppressor cells was demonstrated in a cellular system, graft versus host (GVH) disease. This represents the first example of suppressor cells in a model of cell mediated immunity. Six

month old NZB/W spleen cells induced a vigorous GVH response. This was significantly suppressed by thymus cells from 6 week old NZB/W mice.

4. The role of thymic suppressor cells in the autoimmune disease of New Zealand mice was more directly demonstrated by grafting 2 week or 10 week old NZB/W thymuses into neonatally thymectomized NZB/W mice. The 2 week old, but not the 10 week old thymus grafts prevented the acceleration of autoimmune disease induced by neonatal thymectomy. Repeated grafting of young thymuses into a group of NZB/W mice as they aged led to an increase in survival.

Significance to Bio-Medical Research and the Program of the Institute:

These studies suggest that New Zealand mice lose thymic suppressor cells as they age leading to hyperactive antibody responses including autoantibodies. In teh absence of normal thymic regulatroy function, B cells respond to self antigens. Furthermore, with an expanded B cell proliferation the magnitude of such a response to self antigens is especially excessive. Such a model is applicable to dertain human diseases such as systemic lupus. Similarly, loss of suppressor cells in cell mediated immunity may lead to lymphoid infiltration of many organs in New Zealand mice and perhaps a similar mechanism is operative in human diseases such as Sjogren's syndrome, polymyositis, thyroiditis, rheumatoid arthritis. Loss of suppressor cells may also predispose to the development of malignancy.

Proposed Course:

Future studies will attempt to further define and separate different subpopulations of T cells, as well as therelative changes in such populations (helper, suppressor, effector, etc.). A recently described antithymocyte antibody may be responsible for the loss of suppressor cells or the opposite may be operative. We will attempt to define the role of such an antithymocyte antibody; determine whether different subpopulations of T cells have different susceptibilities to the action of antithymocyte antibody (are suppressor cells more easily destroyed?); and study the role of genetic and viral factors in the loss of suppressor cells.

Honors and Awards: None

Publications:

1. Powell DE, Steinberg AD: The pathogenesis of autoimmunity in New Zealand mice. IV. Independent stimulation of antibodies to DNA and RNA. Clin Exp Immunol 12:419, 1972.

2. Chused TM, Steinberg AD, Talal N.: The clearance and localization of nucleic acids by New Zealand and normal mice. Clin Expt Immunol 12:465, 1972.

3. Parker LM, Steinberg AD: The antibody response to polyinosinic·polycytidylic acid. J Immunol 110:742, 1972.

4. Scher I, Frantz.M, Steinberg AD: The genetics of the immune response to a synthetic double stranded RNA in a mutant CBA mouse strain. J Immunol, 110:1396, 1973.

5. Gelfand MC, Steinberg AD: Cell synergy in skin graft rejection in NZB/W mice. Transplantation Proceedings 5:145, 1973.

6. Gelfand MC, Steinberg AD: Mechanism of allograft rejection in New Zealand mice. I. Cell synergy and its age-dependent loss. J Immunol. 110:1652, 1973.

7. Chused TM, Steinberg AD, ParkerLM: Enhanced antibody response of mice to polyinosinic·polycytidylic acid by anti-thymocyte serum and its age-dependent loss in NZB/W mice. J Immunol, in press.

Serial No. NIAMDD-ARB- 13c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Therapeutic studies in New Zealand mice

Previous Serial Number:

Principal Investigator: Dr. Alfred Steinberg

Other Investigators: Dr. Michael Gelfand (NIAID), Dr. John Hardin (NIDR), Dr. Peter Scher (RBBH, Boston)

Cooperating Units:

Man Years:

Total:	1
Professional:	1/4
Other:	3/4

Project Description:

Objectives:

New Zealand mice, especially the NZB/NZW F_1 spontaneously develop antibodies to nucleic acids and die of immune complex glomerulonephritis. These mice are an excellent model for comparing different immunosuppressive drug programs. In addition, as the immunologic basis for the disease becomes better understood, they offer a model for studying specific immunotherapy.

Methods Employed:

Comparison of different immunosuppressive drugs given daily singly or in combination upon antibodies to DNA (measured by the ammonium sulfate precipitation assay), proteinuria, kidney histology and survival. Comparison of daily versus intermittent therapy.

Major Findings:

1. Intermittent therapy of female NZB/W mice was superior to daily therapy despite using a lower total drug dosage.

2. Treatment of female NZB/W mice with 3 drugs (cyclophosphamide + azathioprine + steroids) led to significantly less immunofluorescent staining of complement in their kidneys than when treated with 1 or 2 drugs. A similar

reduction in γ_2 but not γ_1 staining was noted.

3. It has been shown that measures of renal function, such as proteinuria, are of more importance in selecting animals for therapeutic studies than is the chronological age of a particular mouse.

4. Immunosuppressive treatment of NZB/W mice with proteinuria > 300 mg% did not lead to increased survival and in some instances was associated with decreased survival secondary to infection.

Significance to Bio-Medical Research and the Program of the Institute:

These studies are designed to provide background experience for clinical studies in which the immunosuppressive drugs may be given in a more effective manner to humans with SLE.

Proposed Course:

Conclude studies defining effects of different immunosuppressive programs and attempt more specific immunotherapy.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-14c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The removal of bilirubin and other albumin bound substances from blood

Previous Serial Number: None

Principal Investigator: Dr. Paul H. Plotz

Other Investigators: Mrs. Kay Gordon

Cooperating Units: Metabolism Branch NCI
Dr. Bruce Scharschmidt
Dr. Paul Berk
John Vergalla
Jean Waggoner

Man Years:

Total:	3/4
Professional:	1/4
Other:	1/2

Project Description:

Objectives:

Substances such as bilirubin that binding tightly to plasma protein cannot be removed by dialysis. We have attempted to remove such substances by employing affinity chromatography on protein - agarose beads.

Methods Employed:

Human albumin is conjugated to agarose: the resulting beads are placed in a column and plasma or whole blood, are passed over the column. Bilirubin or other substances are assayed before and after passage. The bound material is eluted and assayed.

Major Findings:

Agarose-albumin columns can bind in excess of 150 μg Bilirubin per gram of wet weight of gel. The BR can be completely eluted and the gel reused. The gel material is compatible with blood and can be used in a perfusion system with living rats. Thyroxine and salicylate bind to such beads too.

Significance to Bio-Medical Research and the Program of the Institute:

Such conjugates may be useful for removing bilirubin and other protein-bound toxic substances from human blood.

Proposed course:

Further studies will include investigation of the binding of other substances to agarose-protein beads and the preparation of materials safe for in vitro use.

Honors and awards: None

Publications: Abstract in JCI (in press)

Serial No. NIAMDD-ARB-15c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the Combining Sites of Mouse Myeloma Proteins which Bind Phosphoryl Choline

Previous Serial Number: ARB-24c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Bruce Chesebro, Dr. Nortin Hadler and Dr. Dennis Carson

Cooperating Units: None

Man Years:

Total:	1-3/4
Professional:	1-1/4
Other:	1/2

Project Description:

Objectives:

One of the most common activities found among mouse myeloma proteins is the ability to bind phosphoryl choline or its analogues. There already exist 6-8 such proteins which share this property but which are clearly distinguishable on the basis of their binding constant and specificity. These proteins therefore seem ideally suited to study structural functional relationships in immunoglobulin combining sites. Furthermore, since the ligand phosphoryl choline is quite different than the aromatic ligands which have been most frequently studied in immunochemistry some new features of the combining sites of these proteins may become evident.

Methods Employed:

To begin our work it was necessary to purify the proteins and to obtain a suitable labeling reagent. We were able to accomplish both by synthesizing the compound pNO_2 phenyl phosphoryl choline (PNPC) a compound for which no reproducible synthesis had heretofore existed.

Major Findings:

In the previous report we mentioned that the major labeled tyrosine in affinity-labeled TEPC 15 was a tyrosine at a position in the light chain analogous to that labeled in the very different MOPC 315 protein. We also mentioned that some labeling appeared to be taking place at an additional residue. This labeled residue has now been identified as Tyr No. 92. This position is in the most variable portion of the light chain. Because of this variability this region had previously been assumed to be important in defining antibody specificity. Our study provides direct evidence for this proposal and suggests that amino acid side chains from this region make direct contact with the antigen.

As an adjunct to this work we have attempted to define the relative functional importance of the two tyrosines. The procedure was as follows: Affinity labeled (100% inactivated) TEPC 15 was reduced with dithionite in order to cleave the azo bond between the labeling reagent and the modified tyrosines. This causes the conversion of the labeled tyrosines to 3-amino-tyrosines. The activity of the protein was then assessed. Approximately 25% of the molecules appeared to remain totally inactivated. The other 75% appeared to have recovered their normal activity. It is interesting that approximately 75% of the labeling occurs at Tyr_{32} and 25% at Tyr_{92}. Together these results suggest that Tyr 92 is more critically involved in the antigen combining activity.

Affinity labeling of several other anti-phosphoryl choline mouse myeloma proteins was attempted, with the following results: a) Protein 511 which has the lowest K_A for phosphorylcholine was not significantly labeled. b) Proteins 167 and 603 became specifically labeled but the reaction was not as vigorous as with TEPC 15. While TEPC 15 becomes labeled exclusively on the light chains , 167 and 603 become labeled predominantly on the heavy chains. c) Protein HOPC 8 is believed to be identical to TEPC 15 by a variety of criteria even though it arose independently. We extensively documented the labeling of this protein by our labeling reagent and found it to behave identically as TEPC 15. These results give additional weight to the proposal that the two proteins are identical. That two myeloma proteins of identical structure can arise independently raises important questions about how antibody synthesis is regulated.

Significance to Bio-Medical Research and the Program of the Institute:

The significance of studying functional myeloma proteins has been discussed in previous Progress Reports. As indicated in the introduction the particular set of proteins should be unusually instructive as far as studying immunoglobulin combining sites.

Proposed Course:

We hope to separate TEPC 15 molecules which have been labeled and reduced and which are active from those molecules which remain inactivated. The two fractions will be characterized with respect to how the label is distributed. In this way we will look for direct evidence for the proposal made in paragraph two of Major Findings.

We plan to continue work on MOPC 603 since the Fab fragments of this protein are crystalizable and an x-diffraction analysis of the fragments is at present underway. This could then be the first protein where one would be able to specify directly the location of the label with respect to the combining site.

Honors and Awards: None

Publications:

Chesebro B, Hadler N, Metzger H: Affinity Labeling Studies on Five Mouse Myeloma Proteins which bind Phosphorylcholine. Proc. Third International Convocation of Immunology. 1972 (In press).

Serial No. NIAMDD-ARB-16c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunoglobulins on Cell Surfaces

Previous Serial Number: 17-c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Karl Becker, Dr. Philip M. Grimley, Dr. Teruko Ishizaka and Dr. Kimishige Ishizaka

Cooperating Units: Section on Pathological Anatomy, NCI and Departments of Medicine and Microbiology, Johns Hopkins University School of Medicine, Good Samaritan Hospital, Baltimore, Maryland.

Man Years:

Total:	1-3/4
Professional:	1-1/4
Other:	1/2

Project Description:

Objectives:

Essentially all the important activities of antibodies are ultimately expressed not in free solution but on cell surfaces. For example antibodies act as antigen receptors on cells destined to respond in the immune response. Similarly it is γE on effector cells which when stimulated by allergens, leads to histamine release. Specific antigen catabolism similarly must be mediated by antigen antibody complexes interacting with phagocytic cells. We are now establishing a variety of experimental situations whereby we can look at such systems.

Methods Employed:

Our initial efforts will be directed to making highly specific anti-immunoglobulin. By employing ferritin labeled antibody reagents and electronmicroscopy we hope to gain a better understanding of the distribution of such immunoglobulin receptor molecules. Recently we have in addition used fluorescein tagged antibodies for our studies.

Major Findings:

We have been able to make "hybrid" antibodies from sheep anti-IgG and anti-ferritin and to improve our yields. This has made our electronmicroscopic studies considerably easier since such hybrid antibodies can now be made in plentiful supply.

We have confirmed that the phenomenon of redistribution of surface IgE on basophils in the presence of anti-IgE is very similar to the same phenomenon described for surface redistribution of immunoglobulins by antibodies. Divalent antibodies produce such redistribution while monovalent Fab fragments do not. Since the latter do bind perfectly well it must mean that "bridging" or cross-linking of the surface IgE is essential. The extent of redistribution is dependent upon the dose of anti-immunoglobulin used, the temperature during the incubation with antibody, and time. It is independent of Ca^{++} or Mg^{++} concentration and of the "responsiveness" of the cell (see paragraph 4).

We have shown conclusively that histamine release is not dependent on redistribution. Evidence for this point is a) histamine release induced by anti-IgE treatment of cells is optimal at doses of anti-IgE which do not cause redistribution of the surface IgE - indeed doses of anti-IgE which are optimal for redistribution produce an inhibition of histamine release; b) histamine release occurs prior to redistribution at doses of anti-IgE which redistribute surface IgE slowly; c) antigen induced histamine release is unaccompanied by redistribution of the surface IgE.

We have shown that many of the biochemical processes which are critical for histamine release do not affect the redistribution of surface IgE; a) redistribution occurs on cells which are relatively unresponsive, i.e., cells which don't release histamine despite otherwise optimal conditions; b) redistribution occurs normally in the absence of free divalent cations in the medium whereas histamine release is totally inhibited; c) redistribution though temperature dependent occurs more or less rapidly even at low temperatures (<25°C). Histamine release is essentially completely inhibited at such temperatures.

Significance to Bio-Medical Research and the Program of the Institute:

Although our major findings are in a sense "negative" ones they have conclusively ruled out one important possible mechanism by which the cells in question are "triggered". As discussed in the previous report the question of how cells bearing immunoglobulin receptors are stimulated is of fundamental importance in studies on the activation of the immune system in health and disease.

Proposed Course:

Although redistribution has been shown not to be important for histamine release in this system, bridging of surface IgE clearly is required. We are at present exploring various other systems in which the consequences of bridging can be explored further.

Honors and Awards: None

Publications:

Becker KE, Ishizaka T, Metzger H, Ishizaka K, Grimley P: Surface IgE on Human Basophils During Histamine Release. (Submitted for publication).

Serial No. NIAMDD-ARB-17c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Receptors for IgE on Mast Cell Plasma Membranes

Previous Serial Number: 18c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Anthony Kulczycki, Jr.
Dr. Chaviva Isersky-Carter

Cooperating Units: None

Man Years:

Total:	2-3/4
Professional:	2-1/4
Other:	1/2

Project Description:

Objectives:

IgE is a unique class of immunoglobulin which plays a predominant role in the physiological events associated with a variety of allergic phenomena. The immunoglobulin becomes attached to the surface of basophils and mast cells and upon exposure to the allergenic antigen mediates the release of histamine and other vasoactive amines from the cells to which it is attached. Although there is an increasing amount of information concerning the biochemistry of this reaction almost nothing is known about how the IgE acts as the cell surface transducer. The present project is directed towards increasing our understanding of how the IgE interacts with mast cells.

Methods Employed:

Rats and mice will be immunized according to protocols which are known to yield relatively high levels of reaginic (IgE) antibodies. In vitro and in vivo methods of assaying these antibodies will be employed. Active sera will be absorbed with the cells from two known mouse mastocytomas. If there is a reduction in the reaginic titre after such absorptions will provide preliminary evidence for receptors for IgE on these cells. The absorption studies will then be followed by more direct investigations of IgE binding e.g. by fluorescent antibody techniques and/or by rosette formation between the IgE coated cells and red cells coated with the antigen to which the IgE is directed. Should these experiments be positive we will then try to isolate mast cell membranes from these cells as a first step to identifying the receptor responsible for IgE binding.

Major Findings:

During the course of this study Minnard and Levy briefly reported similar experiments in which they successfully depleted reaginic activity from mouse sera by incubation with 1 of the mouse mastocytomas employed in our own studies. We have repeated these studies using mouse or alternatively rat reagins and have been able to confirm their results. The next step will be to demonstrate directly that the IgE antibodies become bound to the surface of these cells. This has so far proven difficult. We found that when such cells were incubated with the antisera specific antigen binding could be demonstrated. However, the antibody which was bound was readily removed by washing the cells. This is not the case with IgE bound to normal cells. This result could be due to a) that the affinity of the cells for IgE is very low b) that most of the antigen-binding activity of the treated cells is in fact due to IgG antibodies. The latter are known to bind weakly. The studies so far have been hampered by the unavailability of purified mouse and/or rat reagins and specific antibodies directed to them.

Significance to Bio-Medical Research and the Program of the Institute:

It is of considerable importance to study the behavior of antibodies on the surface of cells in order to more completely understand the mechanism of the immune response. It is on the cell surface that most of the critical steps of the immune response transpire and it is here that one must direct one search for the ultimate mechanisms by which antigen-antibody reactions initiate specific cellular responses.

Proposed Course:

We are presently developing methods to purify rodent reagins and to produce specific antibodies. In addition other mast cell tumors are being explored.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB- 18c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Infection in Systemic Lupus Erythematosus

Previous Serial Number: 13c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Parker J. Staples (Strong Memorial Hospital), Dr. Robert S. Gordon (A-CI), and Dr. Dale Gerding (CR-OD)

Cooperating Units: Strong Memorial Hospital, Rochester, New York; NIAMDD, Clinical Investigations; Division of Computer Research and Technology, Office of the Director

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

The assemblage of data has been completed.

Proposed Course:

The report detailing the findings is currently in revision.

Serial No. NIAMDD-ARB-19c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Leukopenia in Connective Tissue Disease

Previous Serial Number: ARB-16c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Sheldon Wolff (I-LCI) and Dr. Harry Kimball (I-LCI).

Cooperating Units National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigation.

Proposed Course:

This project has been terminated and led to a publication.

Publication:

Kimball HR, Wolff SM, Talal N, Plotz PH, Decker JL: Marrow granulocyte reserves in the rheumatic diseases. Arthritis Rheum (In press).

Serial No. NIAMDD-ARB- 20c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Delayed sensitivity to hepatitis B antigen (HBAg) in patients with hepatitis

Previous Serial Number: 21C

Principal Investigator: Dr. Paul H. Plotz

Other Investigators: Dr. Saul Agus

Cooperating Units: Digestive & Hereditary Diseases Branch, NIAMDD

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

This project will be discontinued due to negative results in 15 experiments. Will resume experiments in guinea pigs eventually.

Serial No. NIAMDD-ARB-21c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Is tolerance in mice due to opsonization of antigen-sensitive cells?

Previous Serial Number: 15C

Principal Investigator: Dr. Paul H. Plotz

Other Investigators: Mrs. Kay Gordon

Cooperating Units: None

Man Years:

Total:	1
Professional:	1/4
Other:	3/4

Project Description:

After completing a small series of experiments to round out this work, it will be discontinued.

Serial No. NIAMDD-ARB-22c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Enrichment of Antigen-Receptor Bearing Immune Cells

Previous Serial Number: 20C

Principal Investigator: Dr. Paul H. Plotz

Other Investigators: Dr. William E. Seaman

Cooperatine Units: None

Man Years:

Total:	1/2
Professional:	0
Other:	1/2

Project Description:

Despite intensive efforts, we never successfully obtained populations adequately enriched in antigen-receptor-bearing cells. The project will be discontinued.

Serial No. NIAMDD-ARB-23c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cation Transport Defects in SLE

Previous Serial Number: ARB-11c

Principal Investigator: Dr. Nortin M. Hadler

Other Investigators: Dr. John R. Gill (H:CE) and Dr. Jerry Gardner (A:DHDB)

Cooperating Units: National Heart and Lung Institute, Clinical Endocrinology Branch and National Institute of Arthritis, Metabolism, and Digestive Diseases, Digestive and Hereditary Diseases Branch.

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

This project has been terminated and led to a publication.

Publications:

1. Hadler NM, Gill JR, Jr., Gardner JD: Impaired renal tubular secretion of potassium, elevated sweat sodium chloride concentration and plasma inhibition of erythrocyte sodium outflux as complications of systemic lupus erythematosus. Arthritis Rheum 15:515-523, 1972.

Serial No. NIAMDD-ARB-24c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on a Mouse Myeloma Protein Having Antibody Activity

Previous Serial Number: 23c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Nortin M. Hadler

Cooperating Units: None

Man Years:

Total:	1/4
Professional:	1/4
Other:	0

Project Description:

Proposed Course:

This project has been completed; no further studies are planned at present.

Publications:

Nortin Hadler and Henry Metzger: The Modification of Affinity Labeled Antibodies. Immunochem 1973 (In Press).

Serial No. NIAMDD-ARB- 25c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cell Mediated Immunity in Myocarditis

Previous Serial Number: 22c

Principal Investigator: Dr. Kent Salisbury

Other Investigators: Dr. Stephen Gus

Cooperating Units: National Cancer Institute

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

This project was terminated due to the departure of the Principal Investigator.

Serial No. NIAMDD-ARB-26c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Metabolic transformation of certain compounds that may be significant in human health and disease.

Previous Serial Number: 27c

Principal Investigator: Dr. Peter Goldman

Other Investigators: Dr. Sheldon Milstien

Cooperating Units: None

Man Years:

Total:	0
Professional:	0
Other:	0

Proposed Course:

This project has been terminated and has led to a publication.

Publication:

Milstien S, Goldman P: The role of the intestinal microflora in the metabolism of guanidinosuccinic acid. J. Bacteriology (In press).

Serial No. NIAMDD-ARB-27c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Analysis of the effect of diet on the urinary Constituents of normal volunteers

Previous Serial Number: 26c

Principal Investigator: Dr. Peter Goldman

Other Investigators: Dr. Donald S. Young (CC:CP) and Dr. Sheldon Milstien

Cooperating Units: Clinical Center, Clinical Pathology Department

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

This project was terminated with the departure of the principal investigator.

Serial No. NIAMDD-ARB-28c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Characteristics of the Carbon-Halogen Bond in Compounds of Biological Importance

Previous Serial Number: 28c

Principal Investigator: Dr. Peter Goldman

Other Investigators: Mr. Jay C. Unkeless

Cooperating Units: None

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

This project was terminated due to the departure of the Principal Investigator.

Section on Mineral Metabolism

A means has been developed for purifying renal receptors for calcitonin and parathyroid hormone. A series of homogenization and density gradient steps allowed recovery of a fraction of renal membranes enriched with respect to adenyl cyclase, sodium-potassium ATPase and receptors for parathyroid hormone and calcitonin. The receptors were identified by determining binding of radioiodinated hormones to membranes as well as by assays for adenyl cyclase. Preparations of brush borders from renal tubules (kindly provided by Dr. David Neville) are not enriched with respect to hormone-stimulated adenyl cyclase. It is concluded that the plasma membrane fractions purified in our laboratory represent the basal portions of renal tubular cells. Iodinated calcitonin, synthetic of salmon type, binds specifically with high affinity to the membranes. The K_m for binding is approximately 10^{-10} M. It should be possible to develop this system into a highly sensitive radioreceptor assay for calcitonin. It has been proven that receptors for calcitonin in bone and kidney are virtually identical in affinity for a series of calcitonin analogs that vary in affinity over $\sim$ 1000-fold range. The affinity of the calcitonin analogs for receptor sites parallels closely the biological potency of the polypeptide *in vivo*.
[Drs. Heath and Aurbach]

Some suggestive evidence was obtained that microtubules of bone cells might be involved in the physiological response to parathyroid hormone. Colchicine injected into rats causes hypocalcemia and prevents the rise in serum calcium following injection of parathyroid hormone into thyroparathyroidectomized rats. The hypocalcemic effect of colchicine appears to be brought about through suppression of bone resorption.

β-adrenergic Receptor of the Avian Erythrocytes.

The interaction of catecholamines with the β-adrenergic receptor and activation of adenylate cyclase were studied with the plasma membrane of turkey erythrocytes. Interaction of β-adrenergic agonists with receptor in these cells causes a specific stimulation of sodium transport. This effect is mediated through the adenylate cyclase-cyclic 3',5'-AMP (CAMP) system and can be reproduced by adding exogenous CAMP. Stimulation of sodium transport by catecholamines is specifically inhibited by propranolol.

The affinity of isoproterenol for receptor was determined by measuring binding to ^{3}H-isoproterenol to plasma membranes prepared from these cells. The K_m for binding to receptor was similar to the apparent K_m for activation of adenylate cyclase by isoproterenol. Binding of catecholamine to receptor was necessary but not sufficient for activation of adenylate cyclase. The dihydroxyphenyl function of the catecholamine molecule was required for binding as well as for activation of adenylate cyclase. The secondary

alcohol function at the β-carbon, stereoconfiguration at the β-carbon, and an amine function, primary or secondary, at the α-carbon were essential for adenylate cyclase activation but not for specific binding. Those compounds that bound but did not stimulate adenylate cyclase were effective inhibitors of isoproterenol-stimulated adenylate cyclase activity. The β-adrenergic blocker, propranolol, was a potent inhibitor of adenylate cyclase activity but a weak inhibitor of binding. It was concluded that in addition to catechol-specific binding, a further interaction between plasma membrane and hormone is necessary for activation of adenylate cyclase. Thus further interaction appears to involve a site specific for the ethanolamine portion of the catecholamine molecule.
[Drs. Bilezikian and Aurbach]

A series of studies has been carried out utilizing selective venous catheterization and a radioimmunoassay for parathyroid hormone for localization of parathyroid adenomas. A high degree of success has been obtained in localizing these tumors preoperatively. The catheter is passed through the femoral and thence through the major veins to reach the neck where selective samples are taken from each inferior thyroid and superior thyroid vein. Radioimmunoassays show that veins draining parathyroid adenomas frequently contain concentrations of hormone 10 to 20-fold or more greater than the concentration in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in one vein draining the adenoma. The contralateral vein shows concentrations equal to peripheral results. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on each side greater than concentrations in peripheral plasma. Usually in these cases the concentration ratio is not as high as for single adenomas. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by non-parathyroid cancers.
[Drs. Bilezikian, Mallette, Heath and Aurbach, NIAMDD, Drs. Pearson and Doppman, Radiology Department, C.C., and Drs. Powell and Potts, Massachusetts General Hospital]

Specific receptors for glucose in the glucose transport system of the isolated renal tubule have been identified. A procedure was developed utilizing radioiodinated phloridzin to identify transport receptors for glucose. Two receptors for phloridzin were found in isolated renal tubules from the renal cortex. One type of receptor is a high affinity system with a K_m of approximately 5×10^{-7} M that is affected by phenols as well as sugars at very high concentrations. The low affinity-high K system is stable to boiling. It was also proved in contradistinction to results of other workers, that phloridzin indeed is transported by renal tubular cells.
[Drs. Heath and Aurbach]

Clinical Research Section, Metabolic Diseases Branch - Phoenix

The primary focus of attention of the Phoenix Clinical Research Section has continued to be cholesterol gallstone disease, a condition with an extraordinarily high prevalence among the American Indian population. During fiscal year 1972 experiments showing the diurnal variation in biliary lipid composition among both Indians and Caucasians were completed. These data were analyzed, and published early in calendar year 1973. The findings indicate that lithogenic bile (i.e. bile which is supersaturated with respect to cholesterol and which therefore predisposes to the formation or growth of gallstones) is present during nighttime fasting hours, even among normal people. Such bile must therefore not be considered inherently abnormal. (Drs. Grundy, Metzger, and Adler)

Using methods previously described by Dr. Grundy and collaborators for measurement of the hourly rates of biliary lipid secretion by marker dilution principles, a series of studies had been completed which indicated that American Indian women with gallstones had a higher than normal absolute rate of cholesterol secretion in the bile. During FY 1973 these studies were extended to a series of Caucasian subjects with and without radiolucent gallstones, a study done in cooperation with the medical staff of the Maricopa County General Hospital. In this group also, it appeared that those patients with gallstones secrete more cholesterol per unit time than do the control subjects. In addition, there was a difference in the direction of a lower bile acid secretion rate in the gallstone patients, although this difference was not quite statistically significant. Thus, it appears likely that the findings in the more extensive series of Indian subjects are applicable to gallstone disease in the Caucasian population as well. (Drs. Grundy, Bennett, Duane, Adler, and Metzger)

Inasmuch as other investigators had indicated that lithogenic bile tended to return to normal after cholecystectomy, biliary lipid secretion rates were measured in eight Indian subjects before and after elective cholecystectomy for gallstone disease. Contrary to the findings of others, this study indicated that the metabolic defect responsible for the production of abnormal bile is not influenced by cholecystectomy. (Drs. Grundy, Metzger, and Adler)

A long-range study which has been begun during the present year addresses itself to the effect of obesity on biliary lipid excretion. This is occasioned by the widely held clinical impression that patients with gallstone disease are on the average more obese than others. Patients and volunteers with obesity, who are motivated to lose weight, are being recruited and placed on a long-term weight-reducing diet either as inpatients or as outpatients. Biliary secretion rates of cholesterol, bile acids, and phospholipids have been measured before weight reduction began, and will be measured again in as many patients as are able to achieve normal weight. The study is in progress on eleven individuals at this time, but none have yet completed the reducing program. (Drs. Grundy, Bennett, and Bennion)

Because of the recent announcements from Mayo Clinic and other research groups that the feeding of chenodeoxycholic acid results in the disappearance of gallstones, studies have been begun aimed at measuring the effects of bile acid feeding on relative composition and flow rates of biliary lipids. These studies have been carried out primarily on normal Caucasian volunteers recruited from Bethesda, rather than Indians. Results are available on the first five volunteers, and indicate that chenodeoxycholic acid results in a decrease in the absolute secretion rate for cholesterol, whereas cholic acid has no effect. Effects on other biliary lipid parameters are variable and do not approach statistical significance. If this finding is in fact the explanation for the beneficial effect of bile acid feeding in gallstone disease, it will add further strength to the hypothesis that the absolute rate of biliary cholesterol secretion is the most important variable in lithogenesis. (Drs. Grundy, Adler, Bennion, and Duane)

Three experimental approaches to the study of cholesterol and bile acid metabolism as related to gallstone disease have been initiated recently, but are not sufficiently developed at this writing for detailed results to be reported. First, an attempt is being made to measure the absolute rate of biliary lipid secretion in the fasting state. This may be important in view of the previously noted diurnal fluctuations, but cannot be measured by the established marker dilution techniques, since these all involve a constant intraduodenal infusion of liquid food formula. The hypothesis is now being tested that a constant intravenous infusion of Indocyanine Green (a dye quantitatively excreted from the blood by the liver through the bile) could serve as an alternative marker. If this is effective, the absolute flow rate of cholesterol, for example, could be determined by measuring the ratio of cholesterol to green dye in a bile sample. (Drs. Grundy, Bennett, and Duane)

Secondly, since the synthesis of bile acids from cholesterol involves the removal and subsequent oxidation of the last three carbons of cholesterol, it may be that the rate of production of radioactive CO_2 after a patient has received cholesterol-26-C^{14}, would serve as a measure of the rate of bile acid synthesis. Preliminary results with this technique suggest that just as there is a diurnal variation in the lipid composition of bile, there may be a diurnal rhythm in the rate of bile acid production. (Drs. Adler, Grundy, Bennett, and Gordon)

Lastly, an attempt is being made to identify adult onset diabetics who are not absolutely insulin-dependent in order to determine whether careful, as opposed to casual, control of diabetes will have an effect on the lithogenicity of bile. The number of suitable subjects identified so far is too small to permit any conclusions. (Drs. Bennett, Bennion, and Grundy)

An alternative approach to the study of cholesterol metabolism, which does not involve the inconvenience of a duodenal intubation, is through cholesterol balance. All cholesterol which enters the metabolic pool contained in the liver and circulating blood must exit from the body either as fecal cholesterol or bacterial degradation products thereof (fecal neutral sterolds), or as bile acids and their degradation products (fecal acidic sterols). If one knows the dietary cholesterol intake, and can obtain accurate measurements of the fecal neutral and acidic output fractions, one can estimate endogenous cholesterol synthesis by difference. These studies, when applied to Indian and Caucasian women, appear to indicate that the Indian group has a significantly higher rate of endogenous cholesterol synthesis; however, the difference between the two groups may be as much related to overweight among the Indians as to a true racial difference. Two of the Indians had gallstone disease; these two did not appear to be significantly different from their non-afflicted controls. These findings appear to suggest that the difference between Indians and Caucasians with respect to susceptibility to gallstones is perhaps more related to the cholesterol excretory aspect of liver function than it is to internal cholesterol synthesis. (Drs. Grundy and Adler)

An in vitro study of bile obtained at cholecystectomy has been undertaken in hopes of throwing further light on the complex physical chemistry of the mixed micelles of biliary lipids. Gallbladder bile enclosed in a standard cellophane dialysis bag will quickly come to equilibrium with outside solution with respect to non-micellar bile salts, while the cholesterol and phospholipids, as well as the bile salts incorporated in the micellar structures, remain inside the bag. This procedure should give a simple index of the critical micellar concentration for bile salts in the complex water-lipid system of whole bile. The results of the studies so far have shown that the apparent equilibrium concentration of free bile salt is higher than had previously been anticipated. (Drs. Grundy, Duane, and Bennett)

Studies in many laboratories have indicated that control of cholesterol biosynthesis, at least in higher mammals, is exerted at the level of hepatic HMG CoA reductase. It is felt that the measurement of the activity of this enzyme in samples of liver tissue obtained at laparotomy from patients with and without gallstone disease, or with and without other diseases involving disturbances of cholesterol metabolism, may be revealing. As a preliminary project, a study of the level of this enzyme in genetically obese versus control rats has been undertaken. At this time the laboratory methods are in a developmental stage and no results are available. (Drs. Flock and Duane)

Among diseases other than cholelithiasis, which are serious health problems for the western American Indian population, two of the most outstanding are diabetes and alcoholism. Because of recent reports in the literature that American Indians do not metabolize ethyl alcohol as expeditiously as do Caucasians, a detailed study seeking differences in alcohol dehydrogenase levels and in rates of alcohol metabolism between Indian and Caucasian groups has been initiated. A subsidiary objective is to determine whether Indians may differ from Caucasians in their ability to increase rates of alcohol metabolism with habitual use of the drug. A contract has been negotiated with Dr. T. K. Li and the University of Indiana Medical Center for the analysis of alcohol dehydrogenase isoenzymes in liver tissue obtained either at autopsy or during the course of necessary laparotomies among Indian subjects. These will be compared with similar materials obtained by Dr. Li and his co-workers in Indiana. In the second phase of the study, Indian subjects who have a reliable history of consistent use of alcohol are being admitted to the Clinical Research Unit if they are motivated to go through with a period of abstinence. Alcohol tolerance studies are being carried out on such subjects immediately after admission and after a period without alcohol. As of this writing, the methodology has been tested and found to give reproducible results, but the number of subjects on whom data is available is still too small to permit firm conclusions. (Drs. Bennett, Bennion, and Li)

Section on Physiology and Clinical Nutrition

Mechanisms of Secretion of Human Eccrine Sweat Glands. We have followed two major lines of research on sweat gland physiology. In the first, using ^{14}C urea as a tracer, we have been able to demonstrate that that urea in sweat which is in excess of blood urea concentrations appears to be derived from a pool trapped within the skin. The urea in this pool was derived from the blood at an earlier time, and has been simultaneously concentrated and delayed in the skin. The probable mechanism of the increased concentration is evaporation of water. Thus, older theories of sweat gland physiology, most notably the one which attributed the excess urea concentration of sweat to passive reabsorption of water in the sweat duct, should be abandoned.

In the other study, we have investigated the function of eccrine sweat glands in normal volunteers undergoing starvation periods of up to one week. During the fast, when body carbohydrate stores are generally depleted, the glycogen content of sweat glands is preserved, as shown by serial biopsies. The production of lactate by the sweat glands and its secretion in sweat decreases, but only by about 20%. These findings strongly suggest that the glycolytic process is in some way essential to the continued functioning of sweat glands, as virtually all body tissues other than the central nervous system support their energy metabolism during starvation by oxidation of fatty acids and related metabolites.

Total Energy Metabolism and Temperature Regulation. The respiratory monitoring equipment attached to the NIAMDD Metabolic Chamber is still being reinstrumented; significant progress has been made during this year and we have reason to hope that it may be operational during the coming year. The primary use of the Metabolic Chamber has been as a controlled environmental chamber for the studies of sweat gland function described above, and the investigations of temperature regulation done in collaboration with units of NIAID and NICHD. The details of the collaborative investigations are summarized in the other Institutes' reports.

Serial No. NIAMDD-MDB-1 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies in Bone Metabolism

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. John Bilezikian, James Phang and G. Donald Whedon

Cooperating Units: Dr. Mones Berman, Mathematical Research Branch, NIAMD
Metabolism Branch, NCI

Man Years:

Total:	2.25
Professional:	.25
Other:	2.00

Project Description:

Objectives: 1) To investigate the factors influencing mineral storage and loss in demineralizing bone diseases. 2) To investigate the response of the parathyroid gland to rapid changes in blood calcium. 3) To study the influence of phosphate on resorption of bone mineral.

Methods Employed: Metabolic balance studies in patients with disorders of calcium metabolism, noting the effects on nitrogen, calcium, phosphate and magnesium balances of various dietary intakes of calcium, phosphate and magnesium. 2) Estimation of pool sizes, turnover rates, bone deposition rates, and absorption rates of calcium in patients by the oral and intravenous administration of tracer doses of ^{47}Ca.

Major Findings: Same as those reported 1968-'69, 1969-'70, 1970-'71 and 1971-'72.

Honors and Awards: None

Publications:

1. Whedon, G.D.: Effects of weightlessness on mineral metabolism: experience to date. Astronautica Acta 17: 119-128, 1972.
2. Whedon, G.D.: Magnitude of psoriasis as a disease problem. J. Invest. Derm. 59: 2, 1972.

Serial No. NIAMDD-MDB-2 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Study of Parathyroid Hormone: Physiological Regulation of Secretion; Physical, Chemical, and Immunochemical Properties; Biochemistry; and Structure, and Mechanism of Action.

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. John Bilezikian, David Heath, Lawrence Mallette, John Doppman, J. T. Potts, Jr., G. W. Tregear, H. Niall, G. P. Mayer, D. Kronfeld and C. Ramberg. and C. Ramberg.

Cooperating Units: University of Pennsylvania School of Veterinary Medicine and Endocrine Unit, Mass. Gen. Hospital.

Man Years:

Total:	3.00
Professional:	.75
Other:	2.25

Project Description:

Objectives: To prepare purified parathyroid hormone in pilot-plant quantities; to study chemical properties and structure of the hormone; to relate chemical properties and structure to biological activity and to study the mechanism of action of the hormone; to develop a clinically useful test for circulating parathyroid hormone; to study the physiological regulation of secretion of the hormone.

Methods Employed: Pure parathyroid hormone is prepared from phenol extracts of bovine parathyroid glands by solvent fractionation, gel filtration and chromatography on carboxymethylcellulose. Purity is assessed by disc gel electrophoresis, ultracentrifugation, immunodiffusion and immunoelectrophoresis, and recovery of the amino- and carboxyl-terminal amino acids. The hormone is assayed *in vivo* in rats, *in vitro* by measuring hormonal activation of adenyl cyclase, or by a highly sensitive radioimmunoassay. Specific chemical and enzymatic methods are used to cleave the hormone to smaller polypeptide fragments. The polypeptide derivatives are sequentially degraded with exopeptidases and the Edman reaction. Adenyl cyclase is measured by determining conversion of α-^{32}P-labeled ATP to cyclic 3',5'-AMP. Endogenous concentration of cyclic 3',5'-AMP is determined by 1) converting the cyclic nucleotide to ATP and measuring the latter with a ^{32}P-ATP exchange reaction or 2) radioimmunoassay.

Major Findings: Mechanism of Action. Our earlier studies showed that the parathyroid status of animals is an important factor regulating excretion of cyclic AMP in the urine. This finding led to development of the thesis that cyclic AMP is involved in the mediation of parathyroid hormone action. Subsequent work showed that parathyroid hormone *in vitro* activates adenyl cyclase in the kidney as well as in bone, the two organs known to be physiological receptors for action of the hormone. Adenyl cyclase in the kidney is found in two different anatomic zones; adenyl cyclase in the cortex responds specifically to parathyroid hormone whereas that in the medulla responds to vasopressin. Further studies show that virtually all the adenyl cyclase detectable in kidney tissue is found within the renal tubules. This work establishes that the receptor for parathyroid hormone in kidney resides within the renal cortical tubule as had been suspected but not proven by indirect *in vivo* physiological experiments.

Chemistry of Parathyroid Hormone. Application of the "sequenator" method to analysis of parathyroid hormone has allowed deduction of the complete amino acid sequence of bovine parathyroid hormone. The molecule is an 84-amino-acid polypeptide with alanine as the amino-terminal residue and glutamine at the C-terminus. There are two phenylalanines (residues 7 and 34), two methionines (residues 8 and 18), one tryptophan (23) and one tyrosine (43). Previous work from this laboratory showed that the biologically important region of the molecule was located at the amino terminus within a polypeptide smaller than that demarcated by the first aspartate residue. Deduction of the sequence of the molecule allowed chemical synthesis of an amino-terminal biologically active fragment. The tetratriacontapeptide representing residues 1-34 in the hormonal molecule was synthesized by a solid-phase method.

After deblocking and purification the synthetic peptide was tested for biological and immunological activity. The product showed qualitatively all the biological properties, *in vivo* as well as in vitro, of the native molecule. Thus, the protean effects of the hormone on both renal and skeletal tissue can be accounted for by the same limited region of the molecule. It is also implied that the receptor sites for the hormone must be similar in bone and kidney.

Human Parathyroid Hormone. Parathyroid hormone was purified from human parathyroid adenomas. Earlier studies proved that the human hormone is different immunologically from the bovine hormone. Amino acid analyses of the hormone purified from each source indicated approximately eight amino acid substitutions in the human hormone. The amino acid sequence has been completed for the first 37 amino acids at the amino-terminal end of the molecule (Dr. Hugh Niall, MGH, Boston). Based on this analysis, Dr. Geoffrey Tregear (MGH, Boston) synthesized the tetratriacontapeptide representing the amino terminus of the molecule. The product is fully as active in vitro as in vivo as the hormone isolated from human parathyroid adenomas (Drs. Potts, Niall, O'Riordan, Keutmann and Aurbach).

Efforts are continuing to collect large amounts of human parathyroid tissue from which further quantities of human hormone can be purified. With the highly sophisticated techniques now available for sequence analysis, it is projected that if a further 1 to 2 mg of human parathyroid hormone can be isolated in pure form it will be possible to determine the amino acid sequence in the C-terminal region represented by the remainder of the molecule, residues 38-84.

A series of studies has been carried out utilizing selective venous catheterization and a radioimmunoassay for parathyroid hormone for localization of parathyroid adenomas. A high degree of success has been obtained in localizing these tumors preoperatively. The catheter is passed through the femoral and thence through the major veins to reach the neck where selective samples are taken from each inferior thyroid and superior thyroid vein. Radioimmunoassays show that veins draining parathyroid adenomas frequently contain concentrations of hormone 10 to 20-fold or more greater than the concentration in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in one vein draining the adenoma. The contralateral vein shows concentrations equal to peripheral results. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on each side greater than concentrations in peripheral plasma. Usually in these cases the concentration ratio is not as high as for single adenomas. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by non-parathyroid cancers.

Significance to Biomedical Research and Program of the Insitute: Same as for 1968-'69, 1969-'70, 1970-'71 and 1971-'72.

Proposed Course of Project: Same as for 1968-'69, 1969-'70, 1970-'71 and 1971-'72.

Honors and Awards: None

Publications:

1. Bilezikian, J.P., Pearson, K., Powell, D., Ketcham, A.S., Doppman, J.L., and Aurbach, G.D.: Selective venous catheterization of the neck: localization of a parathyroid adenoma drained anomalously by nonthyroidal veins. J. Clin. Endocr. 36: 826-829, 1973.

2. Bilezikian, J.P., Aurbach, G.D., Connor, T.B., Pachas, W.N., Aptekar, R., Wells, S.A., Freijanes, J., and Decker, J.L.: Pseudogout following parathyroidectomy. Lancet I: 445-449, 1973.

3. Marx, S.J., Fedak, S.A., and Aurbach, G.D.: Preparation and characterization of a hormone-responsive renal plasma membrane fraction. J. Biol. Chem. 247: 6913-6918, 1972.

4. Marx, S.J., Powell, D., Shimkin, P.M., Wells, S.A., Ketcham, A.S., McGuigan, J.E., Bilezikian, J.P., and Aurbach, G.D.: Familial hyperparathyroidism. Ann. Intern. Med. 371-377, 1973.

5. Potts, J.T., Jr., Keutmann, H.T., Niall, H.D., Tregear, G.W., Habener, J.F., O'Riordan, J.L.H., Murray, T.M., Powell, D., and Aurbach, G.D.: Parathyroid hormone: chemical and immunochemical studies of active molecular species. In Taylor, S. (ed.): Endocrinology 1971. Proceedings of Third International Symposium London, William Heinemann Medical Books Ltd., 1973, pp. 333-349.

6. Powell, D., Shimkin, P.M., Doppman, J.L., Wells, S.A., Aurbach, G.D., Marx, S.J., Ketcham, A.S., and Potts, J.T., Jr.: Primary hyperparathyroidism. New Eng. J. Med. 286: 1169-1175, 1972.

7. Shimkin, P.M., Powell, D., Doppman, J.L., Marx, S.J., Pearson, K.D., Wells, S.A., and Ketcham, A.S.: Parathyroid venous sampling. Radiology 104: 571-574, 1972.

8. Wells, S.A., Ketcham, A.S., Marx, S.J., Powell, D., Bilezikian, J.P., Shimkin, P.M., Pearson, K.D., and Doppman, J.L.: Preoperative localization of hyperfunctioning parathyroid tissue: radioimmunoassay of parathyroid hormone in plasma from selectively catheterized thyroid veins. Ann. Surg. 177: 93-98, 1973.

Serial No. NIAMDD-MDB-3 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the Chemical Nature and Mode of Action of Thyrocalcitonin.

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. David Heath and James Gavin

Cooperating Units: Clinical Endocrinology Branch, NIAMD
Department of Endocrinology, Mass. Gen. Hospital

Man Years:
Total: 1.25
Professional: .75
Other: .50

Project Description:

Objectives: To study the interaction of calcitonin with its specific receptor target organs.

Methods Employed: The interaction of calcitonin with its receptors has been evaluated by determining adenylate cyclase as well as binding of radioiodinated calcitonin to membranes isolated from renal and skeletal tissue.

Major Findings: A purification procedure for kidney membranes has been developed. It has been shown that the calcitonin receptor from the kidney is concentrated at the corticomedullary junction. The membranes which bind calcitonin and contain calcitonin-sensitive adenylate cyclase show low specific activities for renal brush border enzymes, indicating that calcitonin receptors are localized at an anti-luminal aspect of renal cells or at a site that does not contain brush borders. Utilizing a series of calcitonin analogs, dose response curves were compared for receptors from kidney and bone. The dose response curves in the two tissues are virtually superimposable, suggesting that the calcitonin receptor in the two organs has a similar molecular structure.

Significance to Biomedical Research and Program of the Institute: The current investigations should provide further insight into the structure function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful characterizing hormone receptors present in kidney and bone.

Proposed Course of Project: Further studies are in progress to characterize further the interaction of calcitonin with its tissue receptors. It also will be of interest to solubilize calcitonin receptors and further characterize them.

Honors and Awards: None

Publications:

1. Marx, S.J., Woodard, C.J., and Aurbach, G.D.: Calcitonin receptors of kidney and bone. *Science* 999-1001, 1972.
2. Marx, S.J., Ketumann, H.T., Glossman, H., Woodard, C., and Aurbach, G.D.: Renal receptors for calcitonin: binding and degradation of hormone. *J. Biol. Chem*. In press.

Serial No. NIAMDD-MDB-4 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda,Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Pseudohypoparathyroidism and Related Disorders

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Dr. John Bilezikian

Cooperating Units: None

Man Years:

Total:	1.75
Professional:	.75
Other:	1.00

Project Description:

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. Albright proposed that this disorder was attributable to lack of end organ sensitivity to normally-secreted endogenous parathyroid hormone. Our findings that parathyroid hormone action is likely mediated through activation of adenyl cyclase, led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3',5'-AMP in the urine. Pseudopseudohypoparathyroidism is a variant of the disorder and may occur in the same families with pseudohypoparathyroidism. Pseudopseudohypoparathyroidism is characterized by similar constitutional features but no clinical evidence of hypoparathyroidism. Several other syndromes including Gardner's syndrome, basal cell nevus syndrome, syndrome of calcification of basal ganglia, and vitamin D-resistant osteomalacia have also been reported in the past as resistant to the phosphaturic action of parathyroid hormone. Current studies have been extended to include testing patients in these categories as well.

Methods: Parathyroid hormone purified in our laboratories was sterile-filtered for clinical use through courtesy of the N.I.H. Pharmacy. Radioimmunoassay of parathyroid hormone was carried out as described previously. Measurement of 3',5'-AMP was performed according to the procedure devised in this laboratory.

Major Findings: Approximately 70 cases of this disorder have been described in the literature; we have examined 18 of these to date. In normal subjects, parathyroid hormone causes a 30- to 60-fold rise in urinary excretion of cyclic AMP within 30 minutes or less after intravenous administration of the hormone. A similar response has been observed in idiopathic, hypoparathyroid, surgical hypoparathyroid, and pseudopseudohypoparathyroid subjects (the latter category show the constitutional changes but do not have laboratory evidence of hypoparathyroidism). Parathyroid hormone caused little or no increase in excretion of cyclic AMP in patients with pseudohypoparathyroidism. In one case, studied through the cooperation of a clinical investigator at another hospital, there was a moderate rise in excretion of cyclic AMP; however, the diagnosis in this instance may be incorrect, and as yet we have not had the opportunity of seeing the patient at the Clinical Center. Patients with this disorder who fail to respond to parathyroid hormone showed a similarly defective response after prolonged calcium infusion. Parathyroid hormone was detectable in the plasma of pseudohypoparathyroid subjects before infusion of calcium but not after. This experiment tended to rule out the possibility that an abnormal endogenously secreted hormone immunologically reactive but biologically inert interfered with the action of exogenous parathyroid hormone.

The best evidence to date is that pseudohypoparathyroidism represents a genetic defect inherited as a sex-linked, dominant trait. There have been no documented cases of direct male-to-male transmission of the disease and the sex incidence is approximately 2:1 female-to-male. Several pedigrees have been described wherein pseudophyoparathyroid subjects were progeny of pseudopseudohypoparathyroid parents or vice versa. One family studied at the N.I.H. included a mother with pseudopseudohypoparathyroidism and her daughter with pseudohypoparathyroidism. The daughter failed to respond to exogenous hormone but the mother responded normally. We have also observed that, although the response to pseudopseudohypoparathyroidism is normal to exogenous hormone, there is a significantly increased rare of baseline excretion of cyclic AMP in this group. This observation may be of importance in further delineation of the precise genetic mechanism involved in transmission of the disorder. It appears likely that the cause of the disorder may be the existence of genetically deficient or defective adenyl cyclase in the bone and kidney of these subjects.

There is an association of hypothyroidism with pseudohypoparathyroidism and in a few such cases the hypothyroidism has been attributed to selective deficiency of thyrotropin. It seems possible that hypothyroidism in pseudohypoparathyroidism might reflect an abnormal receptor adenyl cyclase complex in the central nervous system or anterior pituitary analogous to the defect in the receptor for parathyroid hormone in kidney and bone. Three patients with coexistent hypothyroidism and pseudohypoparathyroidism were given thyrotropin-releasing hormone as part of a study designed to localize the site of the thyroid defect. One case showed an abnormally low basal concentration of thyrotropin in plasma, but gave a normal response to thyrotropin-releasing hormone. In the two other cases, a mother and daughter, there were high basal concentrations of thyrotropin in plasma. The mother was tested with TRH and showed the exaggerated response to TSH secretion characteristic of primary hypothyroidism. The defect in the first case appears to be at the level of the hypothalamus or higher. The defect in the second two cases may represent an abnormality of the thyroid receptor for TSH itself.

Patients with Cushing's syndrome, basal cell nevous syndrome, syndrome of calcification of the basal ganglia, and vitamin D-resistant osteomalacia showed normal responses to parathyroid hormone as determined by urinary excretion of 3',5'-AMP. It was concluded that the classical phosphaturic response to parathyroid hormone is neither precise nor sensitive enough to discriminate normal subjects from those refractory to parathyroid hormone.

Kidney tissue was obtained from a patient with pseudohypoparathyroidism who died suddenly with complications of a massive pulmonary embolus. It was demonstrated in vitro that the membrane fraction of this kidney contained adenylate cyclase activity which was stimulatable by parathyroid hormone. Thus, it was concluded that pseudohypoparathyroidism did not represent a total lack of the renal parathyroid hormone-sensitive adenylate cyclase system.

Significance to Biomedical Research and Program of the Institute: Same as reported for 1968-'69, 1969-'70, 1970-'71 and 1971-'72.

Proposed Course of Project: Same as reported for 1968-'69, 1969-'70, 1970-'71 and 1971-'72.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-5 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Study of Hormone-Mediated Solute Transport: Interaction of Hormones with Cell Receptors; Interrelationship between Biogeneration of Cyclic AMP and Active or Facilitated Transport.

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. John Bilezikian, David Heath, Jerry Gardner and Hayden Klaeveman.

Cooperating Units: Digestive and Hereditary Diseases Branch, NIAMDD

Man Years:

Total:	2.00
Professional:	1.25
Other:	.75

Project Description:

Objectives: To isolate and purify specific hormone receptors in particular receptor tissues; to map the function of the receptor against activation of adenyl cyclase; to decipher the putative link between generation of cyclic 3',5'-AMP and solute transport in particular cell systems.

Methods Employed: Polypeptide and catecholamine hormones are purified and iodinated by standard methods or methods developed in the laboratory specifically for that purpose. Rate of association of hormones is determined by incubating plasma membrane fractions or whole cell with labeled hormones. The kinetics of binding is determined by rate measurements as well as assessment of steady state conditions. Standard mathematical analyses of results are performed utilizing double reciprocal or Scatchard plots. Specific hormone binding is defined in terms of dissociation constant (K_m) of the system. Physiologically significant binding shows K_m's compatible with the dose-response relationship recognized for the hormone at the physiological level.

Major Findings: Specific receptors for glucose in the glucose transport system of the isolated renal tubule have been identified. A procedure was developed utilizing radioiodinated phloridzin to identify transport receptors for glucose. Two receptors for phloridzin were found in isolated renal tubules from the renal cortex. One type of receptor is a high affinity system with a K_m of approximately 5×10^{-7} M that is affected by phenols as well as sugars at very high concentrations. The low affinity-high K_m system is stable to boiling. It was also proved,in contradistinction to results of other workers, that phloridzin indeed is transported by renal tubular cells.

Incubation of parathyroid hormone with isolated renal tubules for 30 to 60 minutes leads to increased transport of phloridzin. This increase in transport presumably reflects increased synthesis of the carrier or transport system. There is no evidence in this system for immediate activation of transport.

The interaction of catecholamines with the β-adrenergic receptor and activation of adenylate cyclase were studied with the plasma membrane of turkey erythrocytes. Interaction of β-adrenergic agonists with receptor in these cells causes a specific stimulation of sodium transport. This effect is mediated through the adenylate cyclase-cyclic 3',5'-AMP (CAMP) system and can be reproduced by adding exogenous CAMP. Stimulation of sodium transport by catecholamines is specifically inhibited by propranolol.

The affinity of isoproterernol for receptor was determined by measuring binding to ^{3}H-isoproterenol to plasma membranes prepared from these cells. The K_m for binding to receptor was similar to the apparent K_m for activation of adenylate cyclase by isoproterenol. Binding of catecholamine to receptor was necessary but not sufficient for activation of adenylate cyclase. The dihydroxyphenyl function of the catecholamine molecule was required for binding as well as for activation of adenylate cyclase. The secondary alcohol function at the β-carbon, stereoconfiguration at the β-carbon, and an amine function, primary or secondary, at the α-carbon were essential for adenylate cyclase activation but not for specific binding. Those compounds that bound but did not stimulate adenylate cyclase were effective inhibitors of isoproterenol-stimulated adenylate cyclase activity. The B-adrenergic blocker, propranolol, was a potent inhibitor of adenylate cyclase activity but a weak inhibitor of binding. It was concluded that in addition to catechol-specific binding, a further interaction between plasma membrane and hormone is necessary for activation of adenylate cyclase. Thus further interaction appears to involve a site specific for the ethanolamine portion of the catecholamine molecule.

Significance to Biomedical Research and Program of the Instutute: There are a number of disease states which probably represent abnormalities of hormone receptors or the coupling between receptor, generation of cyclic AMP and physiological response to the hormone. This project represents the possible development of a model system wherein the connection between receptor binding of hormone, generation of cyclic AMP and transport can be investigated. Diseases such as nephrogenic diabetes insipidus or pseudohypoparathyroidism can better be explained once the inner-connecting links between these cellular events is understood.

Proposed Course of Project: Continuing studies are directed towards isolation of the receptors for particular hormones in receptor cells; solubilization and purification of the receptor; analysis of the inner-connecting substance between binding and adenyl cyclase activation; identification of the system activated by cyclic AMP in stimulating transport of solute.

Honors and Awards: None

Publications:

1. Bilezikian, J.P., and Aurbach, G.D.: A beta-adrenergic receptor of the turkey erythrocyte. I. Binding of catecholamine and relationship to adenylate cyclase activity. J. Biol. Chem. In Press.

2. Bilezikian, J.P., and Aurbach, G.D.: A beta-adrenergic receptor of the turkey erythrocyte. II. Characterization and solubilization of the receptor. J. Biol. Chem. In press.

3. Greene, M.L., Marcus, R., Aurbach, G.D., Kazam, E.S., and Seegmiller, J.E.: Hypouricemia due to isolated renal tubular defect. Amer. J. Med. 53: 361-367, 1972.

4. Heath, D.A., and Aurbach, G.D.: Uptake of ^{125}I-phloridzin by tubules isolated from the renal cortex of the rat. J. Biol. Chem. 248: 1577-1581, 1973.

Serial No. NIAMDD-MDB-6 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Total Energy Metabolism and Temperature Regulation: Studies in Health and Disease

Previous Serial Number: Same

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigators : Dr. Ronald H. Thompson and Mr. Del Thrasher

Cooperating Units: Laboratory of Clinical Investigation, NIAID
Reproduction Research Branch, NICHD

Man Years:
Total : 2.75
Professional: 1.00
Other : 1.75

Project Description:

Objectives: To apply the technique of continuous, extended indirect calorimetry (respiratory gas exchange) and a number of related metabolic and physiologic techniques to the clinical study of energy metabolism, a) to elucidate the mechanisms of normal physiologic response, particularly temperature regulation, to various stimuli including climatic stess, b) to study the influence and mechanism of action on metabolism of a variety of endocrine hormone and drugs, and c) to investigate the characteristics of energy expenditure, temperature regulation, and aberrations of metabolic processes in a variety of disease conditions.

Methods Employed: Indirect human calorimetry by means of complete continuous expired air analysis in the Metabolic Chamber, total body heat loss and temperature regulation by thermoelectric and radiometric techniques under controlled environmental conditions, and precise measurement of evaporative and metabolic weight loss.

Accomplishments: Work on the reinstrumentation of the indirect calorimetry apparatus has made progress during this fiscal year. The following phases of reinstrumentation have been completed: 1) dehumidification of main air stream, 2) construction of pressure control valves, 3) programming of teletype unit for recording signals reporting oxygen consumption and CO_2 output. Calibration of pressure control valves and measurement of air way resistance is in progress. Final flow rate adjustments and calibration of detectors remain to be done.

The environmental chamber itself has been in frequent use for studies of sweat secretion (q.v). In addition, the chamber has been used extensively for studying the thermoregulatory response of patients on other services to extremes of heat and cold. Approximately 6 patients with fevers of unknown origin, being studied on the NIAID service, have been handled in the chamber. An additional 99 studies have been done in collaboration with investigators in NICHD who are carrying out research on the nature and causes of anorexia nervosa.

Significance to Bio-Medical Research and Program of the Institute: Same as for 1969-'70, 1970-'71, and 1971-'72.

Proposed Course of Project: It is hoped that during the coming year the indirect calorimeter can be completed, giving the Institute the capability once again of measuring oxygen consumption and CO production in patients and volunteers under essentially physiologic conditions. In addition, the ability to monitor $^{14}CO_2$ in the expired air stream should afford also the possibility for metabolic studies using radioisotopes. It is anticipated that the metabolic chamber will continue to serve for controlled environment studies in a variety of investigations.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-MDB-7 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mechanisms of Secretion in Human Eccrine Sweat Glands

Previous Serial Number: Same

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigators : Drs. Ronald H. Thompson and James W. Benson, Jr. and Mr. Del Thrasher

Cooperating Units : Section on Pathology, NHLI; Surgical Branch, NCI

Man Years:

Total	: 3.75
Professional	: 2.00
Other	: 1.75

Project Description:

Human sweat glands are minute, but have an extraordinarily high secretory capacity compared to their own weight. Therefore, the sweat gland may be a useful model system in the continuing effort to relate cellular metabolism to mechanisms of secretion. In addition, detailed knowledge of the function of sweat glands may be useful in "human engineering," and in elucidating those diseases, like cystic fibrosis,which affect sweat gland function.

Methods and Major Findings: Studies have centered on the use of tracers to investigate the rate of transfer of various labeled substances from blood to the secretion of eccrine sweat glands. We have used the total body washdown method for the collection of sweat solutes in quantities adequate for a wide range of chemical and radiochemical determinations.

The study of the transfer of ^{14}C urea from blood to sweat, described in detail last year, has largely been completed. Several additional normal subjects have been studied, and the results are highly consistent with earlier ones. We have begun to analyze the data by mathematical modelling techniques, and are now largely convinced that a trapped pool of urea in the skin is responsible both for the delay in the kinetics of transfer of urea from blood to sweat, and for the fact that sweat urea concentrations exceed those of blood. The intracutaneous urea pool probably becomes concentrated as a result of insensible evaporation of water. A new line of evidence has been obtained with the cooperation of the NCI Surgical Branch. In two patients undergoing necessary amputation, it has been possible to demonstrate directly that there is a higher level of ^{14}C urea in skin than in muscle two days after the isotopic substance is administered by mouth.

Experiments on the effects of starvation on sweat secretion have been extended to a total of 9 subjects, 4 of whom went without food for a full week. Even at the end of one week of starvation, the lactate content of sweat had fallen by only 20%, and the volume of sweat secreted in response to a standard temperature stimulus was unchanged. Sodium and chloride concentrations increased. Serial skin biopsies, stained with P.A.S. for glycogen and studied by Dr. Max Buja, Section on Pathology, NHLI, show that glycogen stores in sweat gland tissue are well maintained throughout prolonged fasting. Sweating during fasting causes a temporary partial depletion of glycogen but such glycogen is replaced, presumably from other body stores or from gluconeogenesis, while the fasting continues. These findings indicate that in the course of evolution, the supply of carbohydrate to sweat gland tissue has received a high priority in the organization of energy metabolism during fasting.

The study of microdissected surviving human sweat glands has been taken up again by Dr. James Benson. He has made extensive trials of a variety of enzymatic procedures for tissue dissociation, hoping to free the glands of remaining adherent adipocytes, but so far has not succeeded in doing this without impairing the metabolic function of the sweat glands themselves. Because of the results noted above in the starvation study, we hope to be able to measure the response of sweat gland tissue to insulin, but the presence of adherent fat cells remains a major disadvantage.

Proposed Course of Project: During the coming year we hope to be able to study the hormonal response of sweat gland tissue *in vitro* to a variety of agents. These experiments will be undertaken by applying insulin and other hormones to sweat glands even in the presence of adherent fat cells. The fat cells will then be separated from the sweat gland epithelium by treatment with collagenase and other hydrolytic enzymes before the tissue is analyzed. Using fragments of adipose tissue without sweat glands as controls, we hope to be able to ascertain whether or not the sweat gland is essentially independent of insulin in the regulation of carbohydrate uptake.

Significance to Bio-Medical Research and Program of the Institute: The processes whereby exocrine glands elaborate and transport their secretory products are of very general interest and are still imperfectly understood. It is hoped that our studies will throw some additional light on the secretory mechanisms within the sweat gland, which may in turn be useful in interpreting the process of secretion in general. More particularly, since cystic fibrosis, a major area of program concern to NIAMDD, is clearly manifest as an abnormality of sweat secretion, we always consider the possibility that further progress in understanding the secretion of normal sweat glands may throw light on the basic inherited defect of cystic fibrosis.

Honors and Awards: 1972 Stouffer Prize awarded to Dr. Robert S. Gordon, Jr.

Publications:

1. Droller, J.J., and Wolfe, S.M.: Thrombin-induced increase in intracellular cyclic 3',5'-adenosine monophosphate in human platelets. J. Clin. Invest. 51: 3094, 1972.

2. Gordon, R.S., Jr., Gardner, J.D., and Kinzie, J.L.: Low mannitol clearance into cholera stool as evidence against filtration as the source of stool fluid. Gastroenterology 63: 407, 1972.

Serial No. NIAMDD-MDB-8(c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Diurnal Variation in Biliary Lipid Composition: Role in Cholesterol Gallstone Formation

Previous Serial Number: NIAMD-MDB-9(c)

Principal Investigator: Dr. Allan L. Metzger

Other Investigators: Dr. Scott M. Grundy, Dr. Ronald Adler

Cooperating Units: None

Man Years:

Total	1
Professional:	1/4
Other:	3/4

Project Description: Project was completed in June 1972. The data were subsequently analyzed and prepared for publication.

Publications:

1. Metzger, A.L., Adler, R., Heymsfield, S., and Grundy, S.M.: Diurnal Variation in Biliary Lipid Composition: Possible Role in Cholesterol Gallstone Formation. New England J. Med. 288:333-336, 1973.

Serial No. NIAMDD-MDB-9 (c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Ariz.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biliary Lipid Secretion in Caucasian Subjects

Previous Serial Number: None

Principal Investigators: Dr. Scott M. Grundy and Dr. Peter H. Bennett

Other Investigators: Drs. William C. Duane, Ronald Adler and Allan Metzger

Cooperating Units: Maricopa County General Hospital

Man Years:
Total: 2
Professional: 1/2
Other: 1-1/2

Project Description:

Recent studies from this section have shown that Indian subjects with gallstones have higher biliary secretion rates of cholesterol and lower biliary secretion rates of bile salts than Indians without gallstones. Since gallstones are composed primarily of cholesterol and cholesterol is held in solution in bile by bile salt-phospholipid micelles, these data suggest that the pathogenesis of gallstones in the Indian population is a reduction in the secretion of solubilizer (bile salts) combined with an increased secretion of solubilizate (cholesterol). In order to test the hypothesis that this combined defect is the cause of gallstones in other populations as well as Indians, a group of non-Indian subjects was recruited for measurement of biliary lipid secretion rates. About half of these non-Indian subjects had gallstones as established by oral cholecystogram while the other half had no indication of having gallstones. Biliary lipid secretion rates were determined in both gallstone and nongallstone patients by a marker dilution technique previously used in this laboratory. Patients were selected without regard to other "risk factors" that might result in a high biliary cholesterol secretion, e.g., obesity.

Methods Employed: Two groups of patients were studied: 1) 14 non-Indian subjects without evidence of biliary tract disease. 2) 10 non-Indian subjects with gallstones documented by X-ray. The mean age of the two groups was identical. Percent ideal body weight was statistically higher in the gallstone patients than in the subjects without gallstones.

Results: The results show that gallstone patients have a definite elevation of cholesterol secretion over the control group. In addition the gallstone group seemed to have a lower rate of bile salt secretion than the control group; however, the difference was significant only at the 10% level. Because the gallstone group had a higher mean actual/ideal body weight ratio, it seemed that the high cholesterol secretion rate in this group might in part be accounted for by the obesity of these subjects. This is in agreement with previous studies which have shown an increased incidence of gallstones in obese patients.

Significance: These studies have shown that cholesterol gallstones are associated with a high biliary secretion rate of cholesterol and probably with a low biliary secretion rate of bile salts in non-Indian subjects as well as in Indian subjects. Obesity appears to contribute to the high cholesterol secretion rate thus suggesting an explanation for the previous observation that obesity is associated with gallstone formation.

Proposed Course: This project is now completed and the results are being prepared for publication.

Honors and Awards: None

Publications:

1. Grundy, S.M.: Working Conference on Gallstones. Gastroenterology 63: 201-203, 1972.

Serial No. NIAMDD-MDB-10(c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Output of Biliary Lipids in Southwestern American Indians with Cholesterol Gallstones

Previous Serial Number: Same

Principal Investigator: Dr. Scott M. Grundy

Other Investigators: Drs. Allan L. Metzger, Ronald Adler

Cooperating Units: None

Man Years:
Total: 2-1/4
Professional: 1/2
Other: 1-3/4

Project Description:

Previous studies indicated that American Indian women with cholesterol gallstones have increased biliary secretion of cholesterol and decreased biliary secretion of bile acids as compared to young caucasian women without gallstones. It was suggested that Indian women develop gallstones as a result of a metabolic abnormality in the liver which results in increased cholesterol synthesis and excretion coupled with decreased conversion of cholesterol to bile acids. The resulting bile is supersaturated with cholesterol relative to the solubilizing agents -- bile acids and phospholipids, thus making it possible for cholesterol to precipitate and initiate gallstone formation. If this hypothesis is correct, then the operation of cholecystectomy, while eliminating the site of gallstone formation, should not alter the observed alterations in biliary lipid secretion. As a test of the hypothesis, eight Indian patients who had had biliary lipid secretion rates measured prior to cholecystectomy were restudied one to twelve months post-operatively, in all cases after total recovery from the surgical procedure.

Methods: Hourly outputs of biliary lipids were measured using the previously described marker dilution technique, after placement of a triple-lumen duodenal tube. This technique permitted estimation of hourly outputs of the biliary lipids over a period of 8 to 16 hours during steady-state conditions in which a 40% fat formula was infused into the duodenum. The subjects consisted of one male and seven female Indians with proven cholesterol gallstones, all of whom had been also studied prior to cholecystectomy.

Major Findings: No significant differences between the pre- and post-operative biliary lipid composition or secretion rates of individual lipids could be demonstrated although changes in given individuals were often large.

Significance: Two recently published studies have suggested that cholecystectomy resulted in correction of the hepatic defect leading to formation of bile supersaturated with cholesterol. Our results conflict with these reports and support the hypothesis that the metabolic defect which predisposes the Indian patients to gallstones is not altered by cholecystectomy.

Proposed Course: Further investigations of the effects of cholecystectomy on biliary lipid secretion rates are indicated and will be performed.

Honors and Awards: None

Publications:

1. Grundy, S.M., Metzger, A.L., and Adler, R.D.: Mechanisms of Lithogenic Bile Formation in American Indian Women with Cholesterol Gallstones. The Journal of Clinical Investigation 51:3026-3043, 1972.

2. Metzger, A.L., Heymsfield, S., and Grundy, S.M.: The Lithogenic Index: A Numerical Expression for the Relative Lithogenicity of Bile. Gastroenterology 62:499-501, 1972.

Serial No. NIAMDD-MDB-11 (c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cholesterol and Biliary Lipid Metabolism in Obesity

Previous Serial Number: None

Principal Investigators: Drs. Scott M. Grundy and Peter H. Bennett

Other Investigator: Dr. Lynn J. Bennion

Cooperating Units: None

Man Years:

Total:	1-3/4
Professional:	1/2
Other:	1-1/4

Project Description:

Objectives: The mechanism for the increased synthesis and secretion of cholesterol in patients with gallstones has not been determined. A possible explanation is obesity, since these patients are generally overweight, and obesity has been shown to be associated with increased cholesterol synthesis. This study attempts to determine if weight loss can reverse the increase in cholesterol synthesis associated with obesity, and thereby decrease the amount of cholesterol secreted in bile.

Methods Employed: Cholesterol synthesis rates, biliary lipid composition, biliary lipid secretion rates and bile acid pool size have been determined in obese Indians and caucasians before and after weight loss.

Major Findings: None

Proposed Course: Of the nine Indian and two caucasians thus far able to adhere to the diet, none have yet completed their full weight reduction but all will be reassessed at that time.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-MDB-12 (c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Study of the Effects of Bile Acid Feeding on Biliary Lipids and Cholesterol Metabolism in Non-Indian Subjects

Previous Serial Number: None

Principal Investigator: Dr. Scott M. Grundy

Other Investigators: Drs. Ronald Adler, Lynn Bennion, William Duane

Cooperating Units: None

Man Years:
Total: 1-1/2
Professional: 1/2
Other: 1

Project Description:

Objectives: Studies from other laboratories have shown that oral feeding of chenodeoxycholic acid (CDCA), but not cholic acid (CA), is effective in eliminating or reducing the size of cholesterol gallstones in man. The mechanism of action of CDCA remains unknown, though its administration is associated with a reduced concentration of cholesterol in bile relative to the solubilizing lipids -- bile salts and phospholipids. This effect could be achieved either by reduced biliary secretion of cholesterol or increased secretion of bile acids and/or phospholipids. In view of the reduction of bile acid pool size in patients with gallstones, the latter mechanism has been favored. The objective of the study is to directly examine the effect of CDCA by measurement of the rate of biliary secretion of cholesterol, bile acids, and phospholipids before and after daily administration of this bile acid for one month.

Methods Employed: Cholesterol balance studies and determinations of biliary lipid secretion rates were performed on 5 normal caucasian volunteers without gallstones over a period of three months on a metabolic ward. The study consisted of three one-month periods, beginning with a control period and followed by one month each of CA feeding, 0.5 to 1 gram/day and CDCA feeding 0.5 to 1 gram/day. The order in which the bile acids were given was alternated to give a crossover design. During each period the following parameters were measured: 1) cholesterol synthesis using the balance technique, 2) cholesterol absorption, by simultaneous oral administration of C14 cholesterol and H3 beta-sitosterol followed by measurement of the ratio of C14 to H3 in the neutral sterol fraction of stool samples collected over the subsequent week, 3) bile acid pool size, by measurement of the specific activity of gallbladder bile 12 hours after oral administration of C14 cholic acid, 4) hourly biliary secretion rates of cholesterol, bile acids, and phospholipids under steady-state feeding conditions using a marker dilution technique with a triple-lumen duodenal tube.

Major Findings:

Table 1

	CONTROL (n=5)	CHENODEOXYCHOLIC (n=4)	CHOLIC (n=5)
CHOLESTEROL SECRETION mg/hr ± SE	45.5 ± 6.2	32.6 ± 4.2	46.4 ± 5.6
BILE SALT SECRETION mg/hr ± SE	1345 ± 171	1145 ± 152	1260 ± 172
PHOSPHOLIPID SECRETION mg/hr ± SE	332 ± 82	227 ± 34	271 ± 41
BILE SALT POOL mg ± SE	2744 ± 254	3030 ± 195 (n=5)	3634 ± 333

CDCA feeding was associated with a significant reduction in biliary cholesterol secretion with no change in bile salt or phospholipid output. In every patient in whom cholesterol secretion rate was measured after CDCA feeding, it was lower than in the control period. Cholic acid, in contrast failed to lower cholesterol secretion, although it resulted in greater expansion of the bile salt pool than CDCA. As a consequence of reduced cholesterol secretion the molar concentration of cholesterol in gallbladder bile was reduced after CDCA feeding.

Significance: The results of this study on normal volunteers suggest that chenodeoxycholic acid improves bile composition and promotes gallstone dissolution in cholelithiasis patients by a direct reduction of biliary cholesterol output rather than an increase in output of solubilizing lipids, i.e., bile salts or phospholipids. Whether this effect can be attributed to a reduction of cholesterol synthesis or to shifting of cholesterol into body pools may be answered by the results of the balance studies currently being analyzed. If, in fact, cholesterol synthesis is reduced proportionally to cholesterol secretion, then CDCA could potentially have an important role in the treatment of hyperlipidemia as well as cholelithiasis.

Proposed Course: Additional subjects will be examined using similar methods and with additional parameters of cholesterol metabolism.

Publications:

1. Grundy, S.M.: Treatment of Hypercholesterolemia by Interference with Bile Acid Metabolism. Archives of Internal Medicine 130:638-648, 1972.

Serial No. NIAMDD-MDB-13 (c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Measurement of Biliary Lipid Secretion in the Fasting State

Previous Serial Number: None

Principal Investigators: Drs. Scott M. Grundy and Peter H. Bennett

Other Investigators: Dr. William C. Duane

Cooperating Units: None

Man Years:
Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

Objectives: To determine biliary lipid secretion rates in the fasting state. Recent studies from this laboratory have shown that the composition of bile in the fasting state is much more lithogenic than bile from the same person during feeding. However, measurement of biliary lipid secretion rates during fasting has proven difficult because infusion of a marker into the duodenum results in unpredictable stimulation of gallbladder contraction.

Methods Employed: A technique for the measurement of fasting biliary lipid secretion rates has been developed that does not require the infusion of a marker into the duodenum. This technique involves intravenous infusion of Indocyanine Green at a constant, known rate. By periodically sampling duodenal contents for Indocyanine Green, bile salts, cholesterol, and phospholipids, the secretion rates of the three biliary lipids can be calculated. To establish the validity of this approach, we have measured cholesterol secretion during the formula infusion state simultaneously with Indocyanine Green and with duodenal marker infusion. This should enable us to establish the accuracy and reproducibility of the Indocyanine Green measurement of secretion rate as well as investigate the effects of Indocyanine Green on the biliary lipid secretion rates themselves.

Results: Using this method in a small number of subjects secretion rates of cholesterol were identical when calculated on the basis of Indocyanine Green infusion or on the basis of duodenal marker infusion. We have not demonstrated a definite inhibition of bile secretion by Indocyanine Green, but we cannot say that such an inhibition does not occur.

Proposed Course: More patients will be examined with the methods described above. If these studies confirm that Indocyanine Green is a reliable marker for measurement of biliary lipid secretion rates, about 12 Indian patients will be recruited for measurement of fasting biliary lipid secretion rates. It is likely that a group of caucasian patients will also be recruited to determine any racial differences in fasting biliary secretion rates and a systematic study of the effect of chenodeoxycholic acid feeding on fasting biliary lipid secretion rates will be performed.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-MDB-14 (c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Estimation of Bile Acid Synthesis Rate by Measurement of $^{14}CO_2$ Excretion after Intravenous Cholesterol-26-^{14}C

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Ronald Adler, Dr. Robert S. Gordon

Cooperating Units: None

Man Years:
Total: 1/12
Professional: 1/12
Other: 0

Project Description:

Objectives: Little is known of the factors controlling bile acid synthesis in man. Currently used methods to measure bile acid synthesis require steady state conditions for periods of days to weeks and are not applicable to detection of changes in synthesis over a period of hours or days.

Methods: Bile acid synthesis over short intervals of time could be estimated by monitoring the conversion of cholesterol to bile acid by measurement of $^{14}CO_2$ excretion derived from the three carbon fragment cleaved from the side chain of cholesterol labelled at the C-26 position.

We plan to test the validity of this method in comparison to the balance technique, and to study the factors which normally control the rate of bile acid synthesis.

Major Findings: Pilot studies in one subject indicate evidence of a diurnal variation of bile acid synthesis.

Significance: The availability of a method to monitor bile acid synthetic rates over short time periods would facilitate the study of many factors which may affect these rates and which may be of importance in understanding the pathogenesis and dissolution of human cholesterol gallstones.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-MDB-15 (c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Effect of Diabetic Control on Cholesterol Metabolism

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Lynn J. Bennion and Dr. Scott M. Grundy

Cooperating Units: None

Man Years:
Total: 1/12
Professional: 1/12
Other: 0

Project Description:

Objectives: Because of reports of higher rates of cholesterol cholelithiasis as well as atherosclerosis among diabetics, this study was undertaken to measure basic parameters of cholesterol and biliary lipid metabolism in the controlled and uncontrolled diabetic state.

Methods: Diabetic subjects with poorly regulated carbohydrate metabolism will be studied for a period of one month using the cholesterol balance technique and standard determinations of biliary lipid secretion. Following close regulation of the carbohydrate disturbance the subjects will be reexamined using identical methods.

Major Findings: None. Only two subjects have completed studies up to this time.

Significance: Studies will indicate whether or not diabetic control may be important in the prevention of cholesterol gallstones. The cholesterol balance data may shed some light on why diabetics are prone to atherosclerosis.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-MDB-16(c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Study of Cholesterol Gallstone Formation by the Method of Cholesterol Balance

Previous Serial Number: NIAMD-MDB-10(c)

Principal Investigator: Dr. Scott M. Grundy

Other Investigator: Dr. Ronald Adler

Cooperating Units: Rockefeller University

Man Years:
Total: 3-1/4
Professional: 3/4
Other: 2-1/2

Project Description:

Objectives: Several lines of evidence have suggested the existence of one or more alterations of cholesterol metabolism in Southwestern American Indian women. These include their very high prevalence of cholesterol gallstones, an abnormally high rate of biliary cholesterol secretion as compared to caucasian controls, reduced bile acid pool size, and, from preliminary studies, an increased rate of cholesterol synthesis measured by the cholesterol balance technique. On the other hand, the Southwestern American Indians tend to have, by caucasian standards, very low plasma cholesterol concentrations and a low prevalence of arteriosclerotic cardiovascular disease. These observations suggest that the Indian might be fundamentally different from the caucasian in terms of the relationships between cholesterol synthesis, excretion, and body pools. The elucidation of such a difference would have a profound impact on our understanding of the pathogenesis of atherosclerosis as well as cholesterol cholelithiasis.

Although the studies we previously reported suggested a significant elevation in cholesterol synthesis in Indians with gallstones, this difference could be attributed to the greater age and excess body weight of the gallstone patients. To resolve this difficulty, we have repeated these studies in young Indian and caucasian women more favorably matched in terms of age and weight. In addition to the measurements of cholesterol balance, isotope kinetic studies with labeled cholesterol were carried out in the same volunteers to estimate the size of the body cholesterol pools and the efficiency of absorption of ingested cholesterol.

Methods: The subjects were normal volunteers. They were maintained on the metabolic ward for 4 to 13 weeks. Nine of the volunteers were American Indian women 18 to 19 years old; though they all denied symptoms of biliary tract disease, two of the volunteers were found to have gallstones by oral cholecystogram. The 12 caucasian volunteers consisted of 6 normal weight and 6 overweight 18 to 19 year old college students, none of whom had symptoms referable to the biliary tract. In addition, five Indian women who were employees of the Phoenix Indian Medical Center volunteered for outpatient cholesterol kinetic studies.

1. Cholesterol balance technique: On a solid food diet with 40% of calories as fat and a carefully measured intake of cholesterol and total calories, total fecal output of cholesterol (neutral sterol) and bile acids (acidic sterols) was measured in seven day stool pools for a period of one month, using thin-layer and gas-liquid chromatographic techniques. Outputs were corrected to daily excretion rates by the use of nonabsorbable markers. Throughout the study body weight was maintained within 1-2 kilograms. Cholesterol balance is the sum of the daily fecal output of neutral and acidic sterols minus the dietary cholesterol intake. In the steady state cholesterol balance should represent total cholesterol synthesis.

2. Cholesterol kinetic studies: The decay of plasma cholesterol specific activity was measured for a period of 10 to 13 weeks after administration of 50 microcuries of C-14 cholesterol intravenously to 6 young Indian volunteers, 5 older Indian women, and 5 young caucasian volunteers (two of whom were studied by Dr. R. S. Gordon at Bethesda). Computerized input-output analysis of the resulting decay curves (courtesy of Dr. Paul Samuel of the Rockefeller University) gave cholesterol turnover rate, the magnitude of the total body cholesterol pool, and pool A, the rapidly miscible pool, which is composed of the plasma, red blood cells, liver and bile.

In addition, 9 of these volunteers (8 caucasians, 6 Indian) received 50 microcuries of H3-cholesterol by mouth simultaneously with the intravenous C-14 cholesterol in order to measure the efficiency of absorption of dietary cholesterol. The tritiated cholesterol was given in fatty formula and followed by a meal; measurement of the ratio between H3 and C14 in plasma after a period of several days gave an estimate of the fraction of the dietary cholesterol absorbed. After the third day this ratio remained constant.

Major Findings: The results of the cholesterol balance studies are shown in table 1. The Indian women have a significantly higher rate of cholesterol synthesis (cholesterol balance) than the normal weight caucasian women. However, there is no difference between the Indians and the overweight caucasian women. Furthermore all differences disappear when the results are corrected to 70kg body weight. No differences in the ratio of bile acid to cholesterol excretory products was detected among the different groups. The two Indians with gallstones were not different from the other members of their group. The results for all three groups are comparaable to those reported by other laboratories for caucasian subjects, but are significantly lower than our previous results for older Indian women with gallstones, who were generally overweight.

Table 1 Cholesterol Balance Study
(Mg/day ± SD)

Group	Neutral Sterols	Acidic Sterols	Cholesterol Balance
Uncorrected Values			
A. Indian Women	600 ± 168	235 ± 127	675 ± 290
B. Caucasian Women	374* ± 93	226 ± 83	450* ± 83
C. Obese Caucasian Women	621¶ ± 243	304¶ ± 122	731¶ ± 327
Corrected to 70 KG Body Weight			
A. Indian Women	649 ± 114	256 ± 129	595 ± 201
B. Caucasian Women	454* ± 99	280 ± 60	572 ± 104
C. Obese Caucasian Women	556¶ ± 154	267 ± 79	648 ± 235

*Significantly different from Group A
¶Significantly different from Group B

Results of the cholesterol kinetic studies are shown in table 2.

Table 2 Cholesterol Kinetic Studies
(Mean ± SD)

	Cholesterol Turnover (Mg/day)	Pool A (Gm)	Total Pool (Gm)	Dietary Cholesterol Absorption %
Uncorrected Values				
Indian Women				
Inpatient	883 ± 250	14.6 ± 1.01	56.9 ± 14.1	41.6 ± 8.4
Outpatient	1310 ± 370	19.0 ± 4.72	55.3 ± 18.9	-
Caucasian Women	1130 ± 280	21.3 ± 4.33	60.1 ± 27.8	55.5 ± 21.0
Corrected to 70 KG Body Weight				
Indian Women				
Inpatient	1050 ± 296	17.4 ± 1.20	67.5 ± 16.7	
Outpatient	1190 ± 335	17.2 ± 4.27	50.2 ± 17.1	
Caucasian Women	1040 ± 260	19.6 ± 4.0	55.5 ± 21.0	

When the results for cholesterol turnover and pool sizes were corrected for the larger body mass of the caucasians, all differences disappeared. Measurement of cholesterol absorption by simultaneous administration of C14 cholesterol intravenously and H3 cholesterol by mouth proved to be feasible in terms of a very constant plasma H3 to C14 ratio from 3 days to the end of the study. There was no significant difference between the two groups in whom this parameter was measured, although there were only three volunteers in the caucasian group. The percentage absorption in both groups were similar to results from other laboratories in subjects on diets comparably low in cholesterol.

Significance of these findings: The results suggest that in the age group studied there are no major differences between Indian and caucasian females in terms of cholesterol synthesis, bile acid synthesis, cholesterol pool size, and intestinal cholesterol absorption. Thus it is possible that the previously noted high cholesterol synthesis in Indian women with gallstones could be attributed to their obesity; indeed other laboratories have reported a consistent elevation of cholesterol synthesis, measured by cholesterol balance techniques, in obese subjects. Since the group of young Indian women studied were at great risk of developing cholesterol gallstones in their next decade of life (in fact 2 of the 9 already had gallstones), these results suggest that if a defect in the hepatic metabolism of cholesterol is responsible for the formation of cholesterol gallstones, it is either not detectable by our present techniques or is nonspecifically associated with obesity.

Proposed Course: In view of the fact that other investigators have reported elevated activity of the cholesterol biosynthetic pathway in liver biopsies from patients with cholesterol gallstones, it would seem worthwhile to study cholesterol synthesis by balance or isotope kinetic techniques in a larger group of gallstone patients together with a group of age, sex and weight matched controls. Until such a study is completed, the presence of increased cholesterol synthesis in gallstone patients remains unproven.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-MDB-17 (c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Critical Micellar Concentration of Human Biliary Mixed Micelles

Previous Serial Number: None

Principal Investigators: Drs. Scott M. Grundy and Peter H. Bennett

Other Investigator: Dr. William C. Duane

Cooperating Units: None

Man Years:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: Because of the complexities of mixed micellar systems such as that system which solubilizes cholesterol in bile, little information is available about the critical micellar concentration of human biliary mixed micelles. Measurement of this mixed micellar CMC would allow us to calculate thermodynamic parameters of the mixed micelle, and thus make an estimate of the stability of the mixed micelle under a variety of experimental conditions.

Methods Employed: Traditional methods of measuring the critical micellar concentration of micellar systems cannot be applied to bile because one or the other of the multiple components of bile interfere with these methods.

We have investigated the technique of equilibrium dialysis for measurement of the non-micellar bile salt concentration of bile. This is feasible since the large mixed micelles do not dialyze, but the small bile salt monomers easily dialyze. Because of the very nature of micellar systems, this free bile salt concentration can be shown to be an extremely close approximation of the critical micellar concentration. Three variables have been studied with respect to their effect on this mixed micelle CMC: 1) the micellar bile salt/phospholipid ratio (B/P), 2) ionic strength, 3) dilution.

Results: The free bile salt concentration of 15 gallbladder biles at small dialyzate volumes has been surprisingly high, between 10 and 20 mM/L.

Increasing the dialyzate volume initially lowered the free bile salt concentration considerably without affecting the micellar B/P ratio. At intermediate dialyzate volumes the micellar B/P fell linearly with the falling free bile salt concentration. At large dialyzate volumes the micellar B/P changed very little with a reduction in the free bile salt concentration. Increasing ionic strength resulted in a small, but definite, decrease in the free bile salt concentration. Dilution at constant dialyzate volume results in a lowered free bile salt concentration although such dilutions also result in a reduced micellar B/P. If bile is diluted with B/P held constant, the free bile salt concentration does not change.

Proposed Course: This project is now nearing completion. A few more experiments are indicated to rule out the possibility of a heterogenous population of free bile salt molecules, e.g., some in monomeric form and some bound to a dialyzable protein.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-MDB-18 (c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Hepatic HMG CoA Reductase in Genetically Obese Rats

Previous Serial Number: None

Principal Investigator: Dr. Eunice Flock

Other Investigator: Dr. William Duane

Cooperating Units: Center for Disease Control, Phoenix Laboratories

Man Years:

Total:	1-1/4
Professional:	3/4
Other:	1/2

Project Description:

Genetically obese rats have a characteristic hyperlipidemia with elevated levels of cholesterol as well as triglycerides and phospholipids. An overproduction of very low density lipoproteins in perfused livers of these rats has been reported recently.

The purpose of this investigation is to determine whether the activity of HMG CoA reductase in the liver is altered in these obese rats. This enzyme is known to be rate-limiting for the synthesis of cholesterol and to be rapidly induced after feeding.

Methods: The assay of HMG CoA reductase, as described in the literature involves the isolation of microsomes from liver homogenates, the incubation of the microsomes with ^{14}C- labeled HMG CoA, isolation of the mevalonic acid produced by action of the reductase on the HMG CoA. After conversion to mevalonolactone, it is reported by thin-layer chromatography. The radioactivity of the lactone is measured and the rate of formation of mevalonic acid calculated.

Major Findings: None

Proposed Course: The project is in a preliminary stage while improvements in the assay techniques for HMG CoA reductase are developed.

Significance: The assay of the activity of HMG CoA in obese rats during fasting and feeding should shed some light on the rate of cholesterol synthesis as a possible cause of the accumulation of cholesterol which occurs in the blood.

It is anticipated that the assay techniques used in the rats will be useful for assay of HMG CoA reductase in liver biopsies of subjects as a tool to further investigate the mechanisms leading to the formation of cholesterol gallstones.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-MDB-19 (c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Alcohol Metabolism in American Indians and Caucasians: A Comparative Study of Rates, Mechanisms and Adaptive Capacity

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Lynn J. Bennion, Dr. T. K. Li

Cooperating Units: Indiana University Medical Center

Man Years:
Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

Objectives: Recent studies have shown 1) racial differences in the rate of alcohol metabolism, and 2) a microsomal ethanol ozidizing system (MEOS) of which the significance in humans is as yet undetermined. This project is intended to examine racial differences in alcohol metabolism in Southwest American Indians and caucasians. The rate of alcohol metabolism as well as the role of the MEOS are being investigated.

Methods Employed: The rate of alcohol metabolism is determined by giving an oral dose of ethanol, based on body weight, and following the rate of disappearance of alcohol from the blood over the ensuing 4 and 1/2 hours. Blood alcohol levels are determined using gas-solid chromatography. The activity of the hepatic MEOS is assessed by a) antipyrine clearance tests in the actively alcoholic state, repeated after the subject is in a state of prolonged abstinence from alcohol, and b) alcohol tolerance tests under the same conditions. Antipyrine (a drug metabolized exclusively by hepatic microsomal enzymes, and, like alcohol, distributing itself throughout total body water) is administered orally. Its rate of disappearance from the bloodstream is then followed 8 and 1/2 hours. Plasma antipyrine levels are determined by a spectrophotometric method.

Major Findings: The investigation is still in preliminary stages due to limitations in laboratory personnel and the difficulty of recruiting suitable yet stable subjects. The assays for alcohol and antipyrine have been worked out and appear reproducible and accurate.

Significance: Alcoholism is a major health problem for the Southwest American Indians, and the finding of a racial difference in alcohol metabolism could be relevant to its cause or management. A genetic basis for such a difference has been proposed in the isoenzyme patterns of alcohol dehydrogenase; this question is under study in a companion study undertaken in cooperation with Dr. T. K. Li of the Indiana University Medical Center. The significance of the MEOS in man is as yet uncertain, but may bear upon the problems of adaptation and addiction to alcohol.

Proposed Course: More subjects will be studied using similar methods to ascertain if ethnic differences in alcohol metabolism are present.

Honors and Awards: None

Publications: None

DIGESTIVE AND HEREDITARY DISEASES BRANCH

The work of this Branch is directed toward understanding the origin and treatment of human diseases by examining derangements of underlying processes involving cellular metabolism and physiology. Because the gastrointestinal tract is particularly well suited to pursue this goal and because this organ system lends itself to investigative efforts characterized as being at the interface between basic science and clinical investigation, gastroenterology is the primary focus of this Branch's clinical and laboratory activity. In addition, this Branch has maintained its interest in various inborn errors of amino acid metabolism and transport.

Immunophysiology of the human small intestine

We have previously found that intestinal mucosa from patients with gluten-sensitive enteropathy (GSE) responds to oral ingestion of gluten-containing foods by producing gliadin-specific IgA antibodies. To clarify the role of these antibodies in the pathogenesis of GSE a technique was developed for maintaining biopsy specimens of human small intestinal mucosa in organ culture for up to 48 hours. Using the organ culture technique we have found that intestinal mucosa from patients with GSE in exacerbation produces a soluble factor which will impair biochemical functions and histologic appearance of intestinal mucosa from patients with GSE in remission but not from normal subjects. This ability to manipulate tissue from patients with GSE *in vitro* should greatly facilitate our understanding of the role of the intestinal immune system in GSE. (R. L. Gebhard, Z. M. Falchuk, W. Strober, C. Sessoms and L. Laster)

Biochemical aspects of disease

To characterize *in vivo* the metabolic significance of differences in cystathionine synthase activities in patients with homocystinuria we have initiated studies of nitrogen and sulfur balance. These studies provide a quantitative measure of the relative degree of cystine dependence in a given patient and should enable us to further characterize the differences between pyridoxine-responsive and pyridoxine-nonresponsive patients with homocystinuria. (J. R. Poole, W. A. Edwards, S. Harvey Mudd, and L. Laster)

Studies of membrane function

Virtually every cell in the body is able to maintain a low intracellular sodium concentration and a high intracellular potassium concentration. Although this phenomenon has been studied extensively for the past twenty years we are still ignorant of the molecular structures which are responsible for the transport of sodium and potassium across the plasma membrane. Since cardioactive glycosides specifically inhibit this cation transport mechanism (presumably by inhibiting Na,K-dependent ATPase) we have explored the interaction of cardioactive glycosides with the plasma membrane and have correlated this interaction with the consequent inhibition of membrane cation transport. These studies have enabled us to characterize functionally the optimal condition for membrane-glycoside interactions and should facilitate our ability to isolate and characterize the components of the plasma membrane which are responsible for cation transport. (J. D. Gardner and C. S. Sessoms)

Gastrointestinal hormones

During this past year we have set up a radioimmunoassay for gastrin and are currently developing radioimmunoassays for secretin and cholecystokinin. We also plan to focus on the interactions of each of these three hormones with plasma membranes from hormone-responsive tissues and in so doing begin to elucidate their molecular basis of action. (J. D. Gardner, R. L. Gebhard, W. A. Edwards and H. L. Klaeveman)

Cyclic nucleotide-mediated alteration of cell function

The mechanism by which many polypeptide hormones, as well as catecholamines and cholera toxin, alter cell function appears to be mediated by stimulation of adenylate cyclase and cellular accumulation of cyclic AMP. During this past year we have focused on the mechanism of action of agents which alter cell membrane transport by stimulating adenylate cyclase. These studies have enabled us to demonstrate directly the sequence of steps by which cholera toxin and insulin alter amino acid transport in lymphocytes and by which catecholamines alter cation transport in avian erythrocytes. These studies have also enabled us to explore the initial step in the mechanism of action of these compounds, i.e., their specific interaction with the cell membrane. (J. D. Gardner, H.L. Klaeveman, J. M. Boyle, G. D. Aurbach and I. D. Goldfine)

Serial No. NIAMDD-DHDB-1(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunophysiology of the Human Small Intestine

Previous Serial Number: Same. Previous title "Studies of the small intestine"

Principal Investigator: Dr. Leonard Laster

Other Investigators: Drs. Jerry D. Gardner, Roger L. Gebhard, Hayden L. Klaeveman, NIAMDD; Mrs. Clementine Sessoms, NIAMDD; Dr. Warren Strober, NCI; Dr. Z. Myron Falchuk, Boston, Mass. (formerly NIAMDD); Dr. Stephen Katz, London, England (formerly Dermatology Service, Walter Reed General Hospital); and Dr. A. J. Tousimis (Biodynamics Research Corp.).

Cooperating Units:
National Cancer Institute
Dermatology Service, Walter Reed General Hospital
Biodynamics Research Corp. (Contract NIH 43-66-10)

Man Years:

Total:	2.5
Professional:	1.0
Others:	1.5

Project Description:

Objectives:

The purpose of this study is to investigate characteristic features of the immune system in the human small intestine, and effects of disease on these various processes.

Methods Employed:

Various techniques for studying tissue metabolism were employed. These include the use of isotopically-labeled compounds *in vitro* and *in vivo*, and methods pertinent to enzymology and immunology.

Major Findings:

1. Studies of the immune mechanism in gluten-sensitive enteropathy.

Further refinement of an in vitro model for gluten-sensitive enteropathy (GSE) has revealed that there is a detrimental in vitro effect of gliadin on cultured jejunal tissue from patients with active GSE. This effect is specific for gliadin and is not seen on tissue from GSE patients in remission. This observation suggested the requirement of an "activated endogenous effector mechanism" for the induction of gliadin toxicity. Recent studies suggest that a factor can be transferred in vitro from active GSE tissue to tissue from patients in remission. The immune system is a likely candidate for this toxicity mediator. Immunofluorescent methods are underway in an attempt to further elucidate the mechanism of gluten sensitivity.

2. Relationship of histocompatibility type to small intestinal disease.

Approximately two-thirds of patients with dermatitis herpetiformis (D.H.) have small intestinal villous atrophy resembling GSE (but often assymptomatic) and about two-thirds also have HL-A histocompatibility type 8 (as do 90% of GSE patients). We have studied the relationship between HL-A type and disease in a number of D.H. patients and have found that only D.H. patients with HL-A8 have severe villous atrophy, the majority of D.H. patients with HL-A8 have some degree of villous disease, and the majority of D.H. patients without HL-A8 have normal jejunal histology. Additionally, D.H. patients with intestinal disease synthesize increased amounts of IgA. There also appears to be some bowel, and even skin, improvement in these patients on gluten-free diet. Thus far, however, jejunal biopsy specimens from D.H. patients do not appear to behave in organ culture in the same fashion as biopsy tissue from GSE patients.

3. Studies of patients with "ulcerative jejunitis."

We have studied two patients with ulcerative jejunitis, both with HL-A8 (in common with GSE patients). One patient's biopsy did not behave in organ culture like GSE patients and she subsequently developed lymphoma. Another patient's biopsy did behave like GSE tissue and he appears to show some improvement on steroids and gluten-free diet.

Significance to Biomedical Research and the Program of NIAMDD:

These studies provide information about the role of intestinal immune mechanisms in the pathophysiology of various disease states and about the regulation of biochemical activities of the intestinal mucosa.

Proposed Course:

These projects will be continued and the findings explored further.

Honors and Awards: None

Publications:

Falchuk, Z. M., Rogentine, G. N., and Strober, W.: Predominance of histocompatibility antigen HL-A8 in patients with gluten-sensitive enteropathy. J. Clin. Invest. 51: 1602-1605, 1972.

Katz, S. I., Falchuk, Z. M., Dahl, M. V., Rogentine, G. N., and Strober, W.: HL-A8: A genetic link between dermatitis herpetiformis and gluten-sensitive enteropathy. J. Clin. Invest. 51: 2977-2980, 1972.

Falchuk, Z. M. and Taussig, L. M.: IgA synthesis by jejunal biopsies from patients with cystic fibrosis and hereditary pancreatitis. Pediatrics 51: 49-54, 1973.

Warshaw, A. L. and Laster, L.: Protein-losing enteropathy: Cure by limited ileal resection. Annals Surg. In press.

Warshaw, A. L. and Laster, L.: Protein synthesis by human intestinal mucosa: Variations with diseases of the gut. J. Surg. Res. In press.

Falchuk, Z. M. and Leventhal, B. G.: Budd-Chiari syndrome in a patient with paroxysmal nocturnal hemoglobinuria. Am. J. Dig. Dis. In press.

Serial No. NIAMDD-DHDB-2(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biophysical approaches to the study of human diseases

Previous Serial Number: SAME

Principal Investigator: Dr. Leonard Laster

Other Investigators: Dr. A. J. Tousimis, Biodynamics Research Corp.

Cooperating Units: Biodynamics Research Corp. (Contract #PH 43-66-10)

Man Years: None

Project Description:

The above contract was discontinued September 1972, and the balance of the work on this project has been included under project 1(c).

Serial No. NIAMDD-DHDB-3(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biochemical aspects of disease

Previous Serial Number: SAME

Principal Investigator: Dr. Leonard Laster

Other Investigators: Dr. Jeffery R. Pool and Mr. William A. Edwards, NIAMDD;
Dr. S. Harvey Mudd, NIMH

Cooperating Units: National Institute of Mental Health

Man Years:

Total:	1.75
Professional:	1.0
Others:	.75

Project Description:

Objectives:

The purpose of this study is to examine pathways of metabolism in tissues of the gastrointestinal tract and the disruption of these pathways in disease.

Methods Employed:

Various techniques for studying tissue metabolism were employed. These included the use of isotopically-labeled compounds *in vitro* and *in vivo* and methods pertinent to enzymology and quantitative analytical biochemistry.

Major Findings:

In vitro studies in patients with cystathionine synthase-deficient homocystinuria have demonstrated a small (2-10% of normal) level of enzyme activity in pyridoxine responders, while nonresponders have none detectable. To demonstrate the significance of this small level of enzyme activity, we undertook nitrogen balance studies on cystathionine synthase-deficient patients. Using a low cystine diet and supplemental cystine, we have shown that cystine is not an essential amino acid for responders, although it is for nonresponders, thereby confirming *in vivo* the significance of the *in vitro* findings.

Sulfur balance studies have been conducted on normal volunteers and on patients with homocystinuria while ingesting a diet having a fixed sulfur composition. We have found that a higher intake of essential amino acids is necessary to attain sulfur balance than to attain nitrogen balance, some patients in nitrogen balance being in negative sulfur balance until a higher level of the limiting essential amino acid is supplied. As a result, it is apparent that sulfur balance is a more sensitive indicator of amino acid requirements than nitrogen balance in patients with homocystinuria. Additionally, we have demonstrated a significant difference between pyridoxine responders (while on pyridoxine) and normals on the one hand, and nonresponders on the other, with respect to their ability to convert organic sulfur into inorganic sulfate. The former subjects convert over 80%, while the latter subjects convert less than half of their organic sulfur into inorganic sulfate. This, of course, is presumably due to pyridoxine-stimulated enhancement of the residual cystathionine synthase activity present in responders.

Among those homocystinuric patients classified as pyridoxine responders, studies to relate the degree of their response to varying dosages of pyridoxine hydrochloride are presently being undertaken. Additional forms of potential therapy, both dietary and medical, are being investigated for both responders and nonresponders.

Significance to Biomedical Research and the Program of NIAMDD:

These studies extend our insight into approaches to the therapy of cystathionine synthase deficiency and deepen our understanding of the pathophysiology of the disease. In addition these studies should broaden our knowledge of the general question of intermediary metabolism of sulfur-containing amino acids.

Proposed Course:

This project will be discontinued July 1, 1973.

Honors and Awards: None

Publications:

Volpe, J. J. and Laster, L. Transsulfuration in fetal and postnatal mammalian liver and brain. *Biol. Neonate* 20: 385-403, 1972.

Serial No. NIAMDD-DHDB-4(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies of Membrane Function

Previous Serial Number: 4(c) and 5(c)

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Drs. Hayden L. Klaeveman and Leonard Laster, NIAMDD; Mr. Wm. A. Edwards and Mrs. Clementine S. Sessoms, NIAMDD; Dr. Z. M. Falchuk, Boston, Mass. (formerly NIAMDD); Dr. Ira D. Goldfine, CEB:NIAMDD; Dr. N. M. Hadler (formerly ARB:NIAMDD); Dr. John R. Gill, Jr., NHLI; and Dr. Vincent P. Butler, Jr., Columbia University College of Physicians and Surgeons, N.Y.

Cooperating Units:
Clinical Endocrinology Branch, NIAMDD
Arthritis and Rheumatism Branch, NIAMDD
National Heart and Lung Institute
Columbia U. College of Physicians and Surgeons, N.Y.

Man Years:

Total:	1.75
Professional:	.75
Others:	1.0

Project Description:

Objectives:

1) To characterize functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) to identify the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) to develop techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane; 4) to characterize the mechanism by which the membrane transport of various substrates is altered in certain diseases; and 5) to relate these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

Methods:

By using slices of intact tissue, isolated cells or isolated plasma membranes, and by using radioactive tracers, we have studied membrane binding and transmembrane movement of various substrates. Metabolism of the transported substrate was evaluated by use of techniques appropriate to the substrate being studied.

Major Findings:

Binding of cardiac glycosides to the plasma membrane of human erythrocytes is specific, saturable, temperature-sensitive, can be detected at concentrations as low as 10^{-10}M, depends on the cation composition of the incubation solution, can be blocked by glycoside-specific antibodies and correlates directly with the alteration of cell function.

We have demonstrated in *in vivo* and *in vitro* animal studies that α-aminoisobutyric acid inhibits renal tubular reabsorption of homocystine, but that this inhibition is relatively inefficient, suggesting at least two mechanisms of renal tubular transport of homocystine.

Significance to Biomedical Research and the Program of NIAMDD:

Cellular functions which are localized to the plasma membrane are of fundamental importance in many different areas of human biology so that the significance of these studies is broader than the program of any one institute. Our understanding of disease states which reflect altered membrane function will be intimately dependent on our knowledge of normal membrane function.

Proposed Course:

Studies are in progress to solubilize the plasma membrane glycoside receptor.

Honors and Awards:

A paper entitled "Characterization and solubilization of the digitalis receptor" was selected for presentation at the National Meeting of the American Federation for Clinical Research on April 29, 1973.

A paper entitled "Reversal of the pharmacologic and toxic effects of cardiac glycosides by specific antibodies" was selected for presentation at the symposium of the New York Heart Association, February 1973.

A paper entitled "The isolated thymocyte: a new cell for the study of insulin receptor concentrations" was selected for presentation at the National Meeting of the American Federation for Clinical Research on April 29, 1973.

Publications:

Gardner, J. D., Simopoulos, A. P., Lapey, A., and Shibolet, S.: Altered membrane sodium transport in Bartter's syndrome. J. Clin. Invest. 51: 1565-1571, 1972.

Hadler, N. M., Gill, J. R., Jr., and Gardner, J. D.: Impaired renal tubular secretion of potassium, elevated sweat sodium chloride concentration and plasma inhibition of erythrocyte sodium outflux as complications of systemic lupus erythematosus. Arth. & Rheum. 15: 515-523, 1972.

Taussig, L. M. and Gardner, J. D.: Effects of saliva and plasma from cystic fibrosis patients on membrane transport. Lancet 1: 1367-1369, 1972.

Goldfine, I. D., Gardner, J. D., and Neville, D. M., Jr.: Insulin action in isolated rat thymocytes. I. Binding of ^{125}I-insulin and stimulation of α-aminoisobutyric acid transport. J. Biol. Chem. 247: 6919-6926, 1972.

Falchuk, Z. M., Edwards, W. A., and Laster, L.: Effects of alpha-aminoisobutyric acid on urinary excretion of homocystine in patients with homocystinuria. Metabolism 22: 605-610, 1973.

Gardner, J. D. and Conlon, T. P.: The effects of sodium and potassium on ouabain binding by human erythrocytes. J. Gen. Physiol. 60: 609-629, 1972.

Gardner, J. D., Kiino, D. R., Swartz, T. J., and Butler, V. P., Jr.: Effects of digoxin-specific antibodies on the accumulation and binding of digoxin by human erythrocytes. J. Clin. Invest. In press.

Gardner, J. D. and Kiino, D. R. Ouabain binding and cation transport in human erythrocytes. J. Clin. Invest. In press.

Butler, V. P., Jr., Watson, J. F., Schmidt, D. H., Gardner, J. D., Mandel, W., and Skelton, C. L.: Reversal of the pharmacologic and toxic effects of cardiac glycosides by specific antibodies. Pharmacol. Revs. In press.

Serial No. NIAMDD-DHDB-5(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Gastrointestinal Hormones

Previous Serial Number: None

Principal Investigator: Dr. Jerry Gardner

Other Investigators: Drs. Roger L. Gebhard and Hayden L. Klaeveman, and Mr. William A. Edwards, NIAMDD

Cooperating Units: None

Man Years:

Total:	1.5
Professional:	.75
Others:	.75

Project Description:

Objectives:

1) To develop radioimmunoassays for gastrin, secretin and cholecystokinin; and 2) to explore the mechanism of action of these hormones and their interaction with the plasma membrane of responsive tissues.

Methods Employed:

Standard procedures for radioimmunoassay. Methods used to explore the mechanism of hormone action will depend on the particular tissue studied and the functional alteration produced by the particular hormone.

Major Findings:

During the past year we have developed a radioimmunoassay for gastrin and studies are in progress to develop assays for secretin and cholecystokinin. Appropriate *in vitro* systems are being tested for these hormone responses.

Significance to Biomedical Research and the Program of NIAMDD:

Relatively little is known about the cellular mechanism of action of any of the gastrointestinal hormones. Furthermore, without being able to measure their circulating levels it is impossible to assess their role in various disease states such as esophageal reflux, duodenal ulcer and so-called "irritable colon syndrome."

Proposed Course:

These studies will be continued as specified under Objectives.

Honors and Awards: None

Publications:

Walsh, J. R. and Laster, L.: Enzymatic deamidation of the C-terminal tetrapeptide amide of gastrin by mammalian tissues. *Biochem. Med.* In press.

Serial No. NIAMDD-DHDB-6(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cyclic Nucleotide Mediated Functions

Previous Serial Number: None

Principal Investigator: Dr. Jerry Gardner

Other Investigators: Drs. James M. Boyle and Hayden L. Klaeveman, NIAMDD; Drs. Gerald D. Aurbach and John P. Bilezikian, MDB, NIAMDD; Dr. Robert S. Northrup, Bureau of Biologics, FDA; and Dr. A. J. Tousimis, Biodynamics Research Corporation.

Cooperating Units:
Metabolic Diseases Branch, NIAMDD
Bureau of Biologics, FDA
Biodynamics Research Corp. (Contract #NO1-AM-3-3302)

Man Years:

Total:	2.0
Professional:	1.5
Others:	.5

Project Description:

Objectives:

To characterize the mechanism by which cyclic nucleotides alter cell function. To explore the mechanism of action of agents whose effect on cell function is mediated by cellular accumulation of cyclic nucleotides.

Methods Employed:

Using tissue slices, isolated cell suspension or purified plasma membranes we have used radioactive tracers to study cellular accumulation of cyclic 3',5'-AMP and cyclic 3',5'-GMP and the effects of these nucleotides on cell function. We have also studied the effects of various agents on membrane-bound adenylate cyclase.

Major Findings:

β-Adrenergic catecholamines stimulate sodium and potassium transport in avian erythrocytes and this stimulation is mediated by activation of adenylate cyclase and cellular accumulation of cyclic AMP.

Cholera toxin stimulates amino acid transport in isolated rat thymic lymphocytes and this stimulation involves the sequence of toxin binding to the plasma membrane, stimulation of adenylate cyclase, cellular accumulation of cyclic AMP, stimulation of protein synthesis and stimulation of amino acid transport.

Significance to Biomedical Research and the Program of NIAMDD:

Cellular accumulation of cyclic nucleotides mediates the effect of many agents on cell function. Elucidation of the mechanism by which cyclic nucleotides alter cell function should greatly enhance our understanding of the mechanism of hormone action as well as the pathogenesis of diarrhea observed in cholera.

Proposed Course:

These projects will be continued and explored further.

Honors and Awards:

A paper entitled "The mechanism of action of cholera toxin binding to the cell membrane, stimulation of cyclic AMP and alteration of membrane transport" was selected for presentation at the National Meeting of the American Federation for Clinical Research on April 29, 1973.

A paper entitled "Regulation of Membrane Cation Transport by Catecholamines: Role of adenylate cyclase-cyclic AMP system" for presentation on the main program of the National Meeting of the American Society for Clinical Investigation on April 30, 1973.

Publications:

Gordon, R. S., Jr., Gardner, J. D., and Kinzie, J. L.: Low mannitol clearance into cholera stool as evidence against filtration as the source of stool fluid. Gastroenterology 63: 407-412, 1972.

Gardner, J. D., Klaeveman, H. L., Bilezikian, J. P., and Aurbach, G. D.: Effect of beta-adrenergic catecholamines on sodium transport in turkey erythrocytes. J. Biol. Chem. In press.

ANNUAL REPORT

CLINICAL ENDOCRINOLOGY BRANCH

The branch has enjoyed the collaboration of foreign scientists from Belgium, Hungary, India and Italy. Active staff exchange with the Universities of Naples and Louvain continue and the Branch was very active in the IV International Congress of Endocrinology. There have been no changes in the permanent staff.

I. Thyroid Biochemistry

A. Thyroid Plasma Membrane

The role of purine nucleotides in enhancing TSH activation of thyroid adenylate cyclase was established for TSH, ITP > dGTP > GTP. In contrast, for PGE_1-induced activation these three nucleotides are of equal potency. These effects occur at 0.1 μM concentrations. The stimulation by LATS is also enhanced. Evidence is presented that the TSH and PGE_1 sites on the membranes are different and prostaglandin synthesis is not a prerequisite for TSH stimulation of various thyroid parameters. In addition the binding sites for PGE_1 have been characterized (Wolff, Moore).

B. Thyroxine Synthesis

In further investigations into the coupling reaction as a model for the diphenyl ether formation of thyroxine it was found that the acid hydrate 2,2-dihydroxy-3-oxo-3-(4-hydroxy-3,5-diiodophenyl) proprionic acid is not one of the components of this coupling reaction (Cahnmann, Nishinaga). As expected, coupling was inhibited by the antithyroid agent, methyl-mercaptoimidazole.

C. Iodoproteins

The nature of the primary subunits of thyroglobulin continues to elude definition. Using formic acid a 45,000 MW fragment has emerged as a major component. However, many other bands are present, and this is apparently not due to heterogeneity in the carbohydrate moieties. It has been established that the 27S protein derives directly from 19S without *de novo* protein synthesis (Bilstad, Rall, Salvatore).

D. Thyroid Hormone Secretion

Studies on the role of microtubules in the secretion of the thyroid hormone have continued. Extensive studies have shown the inhibition of secretion by colchicine to be apparently specific for microtubules. Furthermore, if TSH stimulates this secretion through microtubules then this must occur from preexisting subunits which are presumably caused to polymerize and not through *de novo* subunit synthesis (Wolff). The subunit (called tubulin) has a dimeric molecular weight of 115,000 and the monomers of 57,000. The thyroid protein is not abundant and is quite labile as well. Purification to near homogeneity and retention of colchicine binding has progressed satisfactorily (Bhattacharyya, Wolff).

An abnormality in thyroglobulin secretion by a rat thyroid tumor appears to be due to carbohydrate changes in the protein resulting from a deficiency of sialyl transferase bound to particles (Robbins, Monaco).

E. Control of Thyroid Function

The responses of TSH to various physiological stimuli have been investigated in man. Unlike the response to TRH, prolactin and TSH can respond separately in breast feeding women. Antibodies to purified α and β subunits of human TSH have been prepared and the β subunit can be increased in myxedema or after TRH. Increased TSH of myxedema is due both to increased production and decreased removal. Chromatographic differences between pituitary and circulating TSH have been detected (Rosen, Weintraub).

Clinical studies with Li^+ reveal that the decreased T_4 degradation predicted from kinetic models could, in fact, be demonstrated in a number of patients (Carlson, Robbins, Temple). In addition it was found that Li^+ can cause a decrease of the diurnal variation that normally occurs in the release of iodine from the thyroid (Carlson, Robbins).

F. Measurements of Iodocompounds in Biological Materials

The details for anion exchange chromatography for iodoamino acids, thin layer chromatography, and gas chromatography for T_3 and T_4 has been worked out (Lewallen, Cahnmann). Also, a zone electrophoretic method with gel films on slides has been developed for separation of T_4 binding proteins. In addition to the usual 3 proteins, two lipoprotein fractions are also shown to bind T_4 (Lewallen).

G. Thyroxine Protein Interaction

Continued measurements of T_4 binding to prealbumin by means of displacement of 1,8-anilinonaphthalene sulfonic acid (ANS) reveal that the constants derived are a function of the ANS concentration. The binding of ANS molecules for prealbumin was also studied with labeled ANS and yielded the same affinity constant. A satisfactory model to explain the data obtained by equilibrium dialysis, fluorescence and spectroscopy has not yet emerged. The binding of various analogues has also been studied. In particular, a previously unsuspected high affinity of T_3 for prealbumin was detected (Pages, Ferguson, Cahnmann, Robbins).

Studies on the interaction of retinol binding protein with prealbumin were extended. Four, rather than one, binding site for retinol binding protein on thyroxine binding prealbumin were found. In addition, two forms of monkey prealbumin were separated and characterized. They differ in only one tryptic peptide and showed slight physical and thyroxine-binding differences (van Jaarsveld, Robbins, Edelhoch).

H. Mechanism of Hormone Action

Because of the well known role of thyroid hormone in protein synthesis the effect of T_3 on protein kinase activity was investigated. T_3 inhibited cAMP-dependent kinase using histone as substrate (10^{-7}-10^{-4}M) and caused a partial displacement of cAMP from binding protein (McClung, Rall).

In addition two patients with resistance to T_4 and T_3 have been studied. Included in the resistance is the pituitary response to TRF.

II. Polypeptide Hormones

A. Structure

Using a fluorescent probe (ANS), which binds only to native hormone and not its subunit, the rates of dissociation and association of human LH and chorionic gonadotropin have been quantitated under various conditions such as pH and urea, etc. The changes can be used as a chemical assay (Edelhoch, Ingham).

B. Ectopic Hormone Production by Tumors

Specific secretion by tumors both of α and β subunits of human chorionic gonadotropin (hCG) has been established by radioimmunoassay. Cloned cell lines from such tumors differ from each other in that hCG secretion is accompanied by a preponderance of either the α and β subunit (Weintraub, Rosen).

C. Insulin-Receptor Interactions

Insulin receptors have now been characterized in circulating and cultured lymphocytes and in thymocytes. The affinity and specificity properties of these receptors suggest that they closely resemble those of liver and fat cells. This system has been used for a radioreceptor assay and has also yielded a solubilized receptor preparation (Gavin, McGuffin, Archer, Roth, Gorden)

It has been shown, using insulin receptor kinetics, that a number of forms of insulin resistance appear to be due to a loss of receptor sites rather than a change in affinity for insulin. This is true both in man and in obese mice. Partial restoration of receptor sites occurs with diet restriction in obese insulin resistance (Kahn, Soll, Goldfine, Roth, Gorden).

The control of the insulin receptor function in thymocytes was studied in the rat. Hypophysectomy increased and obese hyperglycemia decreased the number of receptors available to insulin. In the liver receptor binding was decreased by treatment with growth hormone and corticosteroids (Goldfine, Soll, Kahn, Roth).

Insulin stimulates DNA synthesis in human fibroblasts in culture without affecting RNA synthesis. The effect occurs by increasing the number of cells synthesizing DNA and may be important in promoting cell growth (Rechler, Wells).

D. Growth Hormone Receptors

Specific growth hormone receptors have been found in cultured lymphocytes and a receptor assay using these cells has been developed (Gavin, Archer, McGuffin).

III. Corticosteroid Secretion

Adrenal tumor cells in permanent culture respond to antimitotic drugs with increased steroid secretion that resembles ACTH-stimulated steroid secretion in all respects (nature of steroid products, dependence on protein synthesis, inhibition by glutethimide, locus before pregnenolone) except for a 6-8 hour latent period and the fact that colchicine and vinblastine act beyond the stage of cAMP generation (Temple, Wolff).

IV. Protein Structure

A. Myeloma Protein (TEPC-15)

Fluorescence enhancement by phosphoryl choline has been used to measure antibody binding affinity and to obtain thermodynamic parameters. Spectral changes obtained with the univalent ab fragment were observed upon hapten binding (Pollet, Edelhoch).

B. Lipoprotein

The molecular properties of a component of high density lipoprotein (Apo Glu-I) have been defined. Although highly structured, the protein is less rigid than native globular proteins. Binding of lysolecithin modifies the protein structure and the native high density lipoprotein is more stable than in its free form (Gwynne, Edelhoch).

C. Histidine Operon

The physical properties of two fused enzymes (covalently-linked) of the histidine operon, aminotransferase and histidine dehydrogenase, have been studied. The enzymatic properties and α-helix content are little altered but the fluorescent behavior of tryptophan and pyridoxal phosphate differs from the wild type (Rechler, Edelhoch).

Serial No. NIAMDD/CEB 1c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Nonenzymic Synthesis of Thyroxine and Thyroxine Analogs

Previous Serial Number: Same

Principal Investigators: H. J. Cahnmann, Ph.D.

Other Investigators: None

Cooperating Units: A. Nishinaga, Ph.D.
Department of Synthetic Chemistry
Faculty of Engineering
Kyoto University
Kyoto, Japan

Man Years:

Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

As previously reported, coupling between diiodotyrosine (DIT) and its keto acid analog 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA) takes place in two steps. While the mechanism of the first step, the autoxidation of DIHPPA to a thyroxine (T_4) precursor (I), is fairly well understood, that of the second step, the reaction of I with DIT to form T_4, is still obscure. A possible intermediate in the second step, the diketo acid hydrate II (R = H) could not be synthesized. It was however, possible to synthesize its ethyl ester (II, R = C_2H_5). This diketo ester proved to be completely unreactive towards DIT from which it was concluded that II is not an intermediate in the second step of the coupling reaction.

2-Mercapto-1-methylimidazole (MMI), a reagent which prevents T_4 formation in tissue slices or in cell-free preparations, was tested for its ability to prevent nonenzymic autoxidative coupling reactions. Since nonenzymic autoxidative coupling of the von Mutzenbecher type proceeds better with diiodophloretic acid (desamino-DIT) than with DIT, the former was incubated aerobically in the presence and in the absence of MMI. Tetraiodothyropropionic acid (T_4-prop) was formed only in the absence of MMI. In some of the reactions in which MMI was absent, 3,3',5'-tricodothyropropionic acid ("reverse" triiodothyropropionic acid) was formed in addition to T_4-prop, presumably due to partial deiodination of the starting material followed by coupling. The formation of 3,3',5'-triodothyropropionic acid is analogous to that of "reverse" triiodothyronine (T_3') in certain nonenzymic model reactions. (T_3' is also formed in vivo but the thyroid hormone/T_3' ratio is considerably higher in vivo than in vitro).

Honors and Awards: Visiting Professorship, Kyoto University, Japan (Awarded by the Japan Society for the Promotion of Science)

Publications:

1. Cahnmann, H. J.: Nonenzymic Model Reactions for the Biosynthesis of the Thyroid Hormones. Annual Report of the Tanabe Amino Acid Research Foundation, Osaka, Japan, 1971.

2. Nishinaga, A. and Cahnmann, H. J.: Model Reactions for the Biosynthesis of Thyroxine. XVII. On the Mechanism of the Conversion of 4-Hydroxy-3,5-Diiodophenylpyruvic Acid into Thyroxine. Chem. Commun., 1973. In press.

Serial No. NIAMDD/CEB 2c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Thyroxine-Protein Interactions

Previous Serial Number: Same

Principal Investigators: R. N. Ferguson, Ph.D., R. A. Pages, Ph.D.,
H. J. Cahnmann, Ph.D., H. Edelhoch, Ph.D.,
J. Robbins, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 3 1/4
Professional: 3
Other: 1/4

Project Description:

Last year's studies on the interaction of thyroxine (T_4) and structurally related compounds, as well as of certain fluorescent dyes with human T_4-binding prealbumin (TBPA) have been extended. Various additional compounds, structurally related to T_4, have been synthesized and purified, then tested fluorimetrically for binding to TBPA by the displacement of bound 1,8-anilinonaphthalene sulfonate (ANS). The thus determined association constants K_L ($L = T_4$ or structurally related compounds) were dependent on the ANS concentration. The molecular size of TBPA remained unaltered in the presence of ANS. This excludes dissociation or association. The observed fluorescence did not change in the course of 10 min. nor did it depend on the order of addition of T_4 and ANS.

Previous experiments have shown that TBPA has two equivalent binding sites for ANS. Since it was desirable to have a direct measure of the association constants K_1 and K_2 for these two sites, and to carry out competitive binding studies in which not only T_4, but also ANS is labeled, tritium-labeled ANS was prepared. Two approaches were investigated for its synthesis. The literature procedure of high temperature reaction of aniline hydrochloride with 8-amino-naphthaline-1-sulfonate in aniline proved to be unsatisfactory. Bromination of ANS gave a product which was identified as tetrabromo-ANS. After preliminary catalytic reductions with deuterium in which a tetradeutero-ANS was isolated, a reduction with tritium was carried out. Experimental conditions in the reductions with dueterium and with tritium were by necessity not identical. In the case of the tritium reduction a much less pure product was obtained which required extensive purification in order to achieve chemical and radiochemical purity. The pure product was then used for a direct measure of TBPA-ANS binding by equilibrium dialysis. The results confirmed that two molecules of ANS are bound to one molecule of TBPA and that $K_1 \approx K_2$ (3×10^5 at pH 7.4).

The binding of both, ANS and T_4, to TBPA was investigated by competitive equilibrium dialysis at pH 7.4 with labeled ANS and unlabeled T_4 and also with $^{125}I\text{-}T_4$ and unlabeled ANS. The results showed a strong dependence of the calculated K_{T_4} on ANS concentration, a problem already encountered in fluorescence studies. Several models for the interaction of T_4, ANS, and TBPA are at present being evaluated for the best fit of the experimental data obtained with the three methods of equilibrium dialysis, fluorescence displacement, and ultraviolet difference spectroscopy.

Tritium-labeled 5-dimethylaminonaphthaline-1-sulfonyl (DNS) - glycine was prepared and its binding to TBPA was studied by equilibrium dialysis. This molecule also had two equivalent binding sites ($K_1 = K_2 = 3 \times 10^5$ at pH 7.4). A fluorescence method analogous to that used for ANS displacement was also used for the displacement of this and other DNS-amino acids. The DNS derivatives, however, proved to be less useful than ANS.

Honors and Awards: None

Publications:

1. Pages, R. A., Robbins, J., and Edelhoch, H.: The Binding of Thyroxine and Thyroxine Analogs to Human Serum Prealbumin. Biochemistry, 1973. In press.

Serial No. NIAMDD/CEB 3c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Analytical Procedures for the Qualitative and Quantitative Determination of Iodoamino Acids

Previous Serial Number: Same

Principal Investigators: H. J. Cahnmann, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1/4
Professional: 1/4
Other: 1/4

Project Description:

In the previously described procedure for the separation of various iodoamino acids by means of small columns of a strong cation exchange resin (H. Ogawara, J. M. Bilstad and H. J. Cahnmann, Biochem. Biophys. Acta 257: 339, 1972) monoiodohistidine (MIH) and monoiodotyrosine (MIT) were only incompletely resolved. This procedure has now been modified in order to permit complete separation of MIH, MIT, diiodohistidine, diiodotyrosine, and iodide. Work is in progress to determine optimal conditions for the separation of these compounds not only from each other, but also from triiodothyronine and from thyroxine.

Honors and Awards: None

Publications:

1. Cahnmann, H. J.: Iodoamino Acids. In Rall, J. E. and Kopin, I. J. (eds.): The Thyroid and Biogenic Amines, Amsterdam, North-Holland Publishing Co., 1972, pp. 27-51. (Vol. I of Methods in Investigative and Diagnostic Endocrinology, ed. by S. A. Berson).

Serial No. NIAMDD/CEB 4c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Structure of Polypeptide and Protein Hormones

Previous Serial Number: Same

Principal Investigators: H. Edelhoch, Ph.D., R. Pollet, M.D., J. Gwynne, M.D., K. Ingham, Ph.D.

Other Investigators: R. Lippoldt

Cooperating Units: R. Canfield, Columbia Presbyterian Hospital
F. Morgan, Columbia Presbyterian Hospital
G. Ross, Child Health and Development

Man Years:

Total: 5 1/2
Professional: 4
Other: 1 1/2

Project Description:

A fluorescence assay for antibody binding affinity has been developed using a myeloma protein, TEPC-15. It was shown that the fluorescence enhancement observed when phosphoryl choline was bound could be utilized to measure the binding equilibrium. The effects of pH and temperature on affinity were determined and thermodynamic parameters were obtained. Measurements are made with 1 μM protein or less and take about a half hour for a complete titration.

In order to observe ligand-induced configurational changes in antibodies the changes in difference absorption, fluorescence, excitation polarization spectra and excitation spectra were obtained with TEPC-F_{ab}[1], the monovalent fragment of the antibody, when it combines with phosphoryl choline. These measurements may indicate that a configurational change in antibody structure occurs on hapten (or antigen) binding.

A chemical assay for human luteinizing hormone (hLH) and human chorionic gonadotropin (hCG) has been developed using the fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS). This probe binds to the native hormones but not to their subunits. Concentrations of hCG and hLH at 2 and 0.2 μM respectively, can be assayed for. The rates of dissociation of native hormone and association of subunits have been quantitatively measured under a variety of conditions (pH, temperature, urea) in order to establish the dependence of the subunit interactions on these variables. More quantitative procedures may now be used to investigate the dissociation of these hormones and the recombination of homologous and hybrid subunits.

The molecular properties of the major protein component (Apo Gln I) of high density serum lipoprotein (HDL) have been studied by fluorescence, absorption, polarization and circular dichorism. By most criteria this delipidated protein appears to be highly structured but not quite as rigid as native globular proteins. Apo Gln I binds to lysolecithin with an affinity of 10^7 M^{-1}. Its molecular structure is modified when bound to lysolecithin, one of the phospholipids of HDL. The behavior of Apo Gln I has also been evaluated in its native form, ie. as a component of HDL where it is found to be more stable than in its free form.

Honors and Awards: None

Publications:

1. Branch, W. T., Robbins, J., and Edelhoch, H.: Thyroxine-binding Prealbumin. Conformation in Aqueous Solutions. J. Biol. Chem. 246: 6011-6018, 1971.

2. Aloj, S., and Edelhoch, H.: The Molecular Properties of Human Growth Hormone. J. Biol. Chem. 247: 1146-1152, 1972.

3. Aloj, S. M., Edelhoch, H., Handwerger, S., and Sherwood, L. M.: Correlations in the Structure and Function of Human Placental Lactogen and Human Growth Hormone. II. The Effects of Disulfide Bound Modification on the Conformation of Human Placental Lactogen. Endocrinology 91: 728-737, 1972.

4. Bilstad, J. M., Edelhoch, H., Lippoldt, R., Rall, J. E., and Salvatore, G.: Isolation and Characterization of Discrete Fragments of 27 S Thyroid Iodoprotein. Arch. Biochem. Biophys. 151: 341-349, 1972.

5. Branch, W. T., Robbins, J., and Edelhoch, H.: Thyroxine-Binding Prealbumin. Conformation in Urea and Guanidine. Arch. Biochem. Biophys. 152: 144-151, 1972.

6. Schneider, A. B., and Edelhoch, H.: Polypeptide Hormone Interaction II. Glucagon Binding to Lysolecithin. J. Biol. Chem. 247: 4986-4991, 1972.

7. Schneider, A. B., and Edelhoch, H.: Polypeptide Hormone Interaction III. Conformational Changes of Glucagon Bound to Lysolecithin. J. Biol. Chem. 247: 4992-4995, 1972.

8. Aloj, S. M., Ingham, K. C., and Edelhoch, H.: Interaction of 1,8-ANS with Human Luteinizing Hormones: A Probe for Subunit Interactions of hCG and hLH. Arch. Biochem. Biophys. 155: 478-479, 1973.

9. Rossi, G., Edelhoch, H., Tenore, A., Van Middlesworth, L., and Salvatore, G.: Characterization and Properties of Thyroid Iodoproteins from Severely Iodine Deficient Rats. Endocrinology 92: 1241-1249, 1973.

10. Salvatore, G., and Edelhoch, H.: Chemistry and Biosynthesis of Thyroid Iodoproteins. In Li, C. H. (ed.): Hormonal Proteins and Peptides, in press.

11. Edelhoch, H., and Schneider, A. B.: Polypeptide Hormone Interaction IV. Conformational Changes of Protected Glucagon Fragments Bound to Lysolecithin. Arch. Biochem. Biophys., in press.

12. van Jaarsveld, P. P., Edelhoch, H., Goodman, D. S., and Robbins, J.: The Interaction of Human Plasma Retinol-Binding Protein with Prealbumin. J. Biol. Chem., in press.

13. Pollet, R., and Edelhoch, H.: The Binding Properties of Anti-Phosphoryl Choline Mouse Myeloma Proteins as Measured by Protein Fluorescence. J. Biol. Chem., in press.

14. Aloj, S., Bruni, C. B., Edelhoch, H., and Rechler, M. M.: Physical Studies Comparing a Genetically Fused Enzyme of the Histidine Operon with Its Component Enzymes. J. Biol. Chem., in press.

15. van Jaarsveld, P. P., Branch, W. T., Edelhoch, H., and Robbins, J.: Polymorphism of Rhesus Monkey Serum Prealbumin: Molecular Properties and Binding of Thyroxine and Retinol-Binding Protein. J. Biol. Chem. in press.

Serial No. NIAMDD/CEB 5c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Measurement of Iodocompounds in Biological Materials

Previous Serial Number: Same

Principal Investigators: C. G. Lewallen, M.D.

Other Investigators: H. Hoch, Ph.D.

Cooperating Units: Veterans Administration Center, Martinsburg,
West Virginia

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Zonal electrophoresis indicates that T_4 is normally carried almost exclusively by three serum proteins which in order of decreasing affinity for T_4 are T_4 binding globulin (TBG), T_4 binding prealbumin (TBPA), and albumin. For the most part interpretation of the data has involved equating the distribution of labeled T_4 among the proteins resolved to the equilibrium partition of T_4 among the same proteins in whole serum. This understandably does not take into account the possibility that the equilibrium distribution in whole serum might be altered by the electrophoretic procedure itself. This consideration is pertinent in view of the fact that in the buffer systems we have studied the mobility of free T_4 may be greater or lesser than that of various binding proteins. This raises the possibility that during electrophoresis T_4 may become separated from a protein to which it was formerly bound. Another consideration is that the role, if any, of the binding proteins in specific cell penetration by T_4 is unknown. Conceivably, if such a process exists, high affinity of the binder for T_4 could be disadvantageous.

These considerations led us to develop several zonal electrophoretic techniques which proved to be capable of demonstrating protein T_4 binders not ordinarily demonstrable by the conventional technique. Agarose gel was used throughout because it provided a convection stabilizing electrophoresis medium to which there was minimal adsorption of free T_4. Of the five techniques developed, only the two most important from the standpoint of technical advantage and findings are described here.

(1) Serum was subjected to zonal electrophoresis in a gel film on a glass slide at either pH 7.4 (Tris maleate) or pH 8.6 (glycine acetate). The moist plate was then uniformly covered for a short time with a solution at desired pH containing T_4-I^{125} and variable carrier T_4, dried, and then autoradiographed. This in effect "stains" the developed protein pattern with radioactive T_4 in a manner analogous to staining a protein pattern with a dye such as brom-phenol blue. It is also possible to electrophorese at one pH, say pH 8.6, to obtain good resolution of components, expose the gel to acid vapor, and then "stain" with T_4 at physiological pH. Normal human serum examined by the technique showed the presence of at least five protein components which bind T_4: TBPA, TBG, albumin, α_1 lipoprotein, and β lipoprotein. In addition there was some indication that the TBPA and albumin zones may each actually be comprised of two components. This technique is quite sensitive, very easy to perform, and especially valuable for weak binders in that the resolved proteins all have equal access to the applied labeled T_4 and are not in competition one with another as is the case in the conventional technique.

(2) Carrier T_4 with or without labeled T_4 was added to hot agarose during gel preparation so as to be uniformly distributed in the gel. Serum was electrophoresed in these gels. The rationale was to avoid the separation of T_4 from weak binders which might be expected to occur in the conventional technique from dissociation of the complex followed by differential migration of T_4 and protein. The patterns were stained or autoradiographed. Protein components were then examined for changes in relative electrophoretic mobility as compared to control runs in gels without added T_4. This technique readily showed appreciable changes in mobility of β lipoprotein and albumin due to added T_4. An increase in anodic mobility is good evidence for anion binding (in this case added T_4) under controlled conditions. For protein components demonstrable by dye staining this technique does not require the use of labeled T_4 to demonstrate T_4 binding.

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 6c
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Analysis of an Intercistronic Region in the Histidine Operon of Salmonella typhimurium; Study of a Fused Enzyme Resulting from Genetic Fusion of Two Adjacent Genes in the Histidine Operon

Previous Serial Number: Same

Principal Investigators: M. M. Rechler, M.D., H. Edelhoch, Ph.D.

Other Investigators: C. B. Bruni, M.D., S. Aloj, M.D.

Cooperating Units: Istituto di Patologia Generale, Universita di Napoli, Naples, Italy

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

A fused enzyme in the histidine operon of Salmonella typhimurium has been purified to homogeneity, and examined enzymatically and physically. The enzymatic properties of one of the component enzymes, an aminotransferase, is not altered in the fused enzyme; the other enzymatic activity, histidine dehydrogenase, shows a slightly altered Km for one substrate and a reduced stability to heat.

A comparison of physical properties of the fused enzyme and the two component wild type enzymes has been undertaken. The α-helical content of the fused enzyme is the sum of the helical contents of the two component enzymes, as determined by circular dichoric activity. The environments of tryptophan residues of the dehydrogenase moiety of the fused enzyme differs from those of wild type dehydrogenase tryptophan residues, based on tryptophan fluorescence. The fluorescence of the pyridoxal-5'-phosphate cofactor of the aminotransferase and fused enzyme is more strongly quenched in the latter protein, and shows a different acid pH dependence. These results suggest structural differences in the aminotransferase and the aminotransferase moiety of the fused enzyme that are not reflected in their enzyme kinetic parameters.

Honors and Awards: None

Publications:

1. Bruni, C. B., Martin, R. G. and M. M. Rechler: COOH-terminal Amino Acid Sequence of Histidinol Dehydrogenase from a *Salmonella typhimurium* Mutant. *J. Biol. Chem.* *247*: 6671-6678, 1972.

2. Rechler, M. M., Bruni, C. B., Martin, R. G. and Terry, W.: An Intercistronic Region in the Histidine Operon of *Salmonella typhimurium*. *J. Mol. Biol.* *69*: 427-452, 1972.

3. Bruni, C. B., Rechler, M. M. and Martin, R. G.: *Salmonella typhimurium* Mutants Lacking Ribonuclease I: Effect on the Polarity of Histidine Mutants. *J. Bacteriology* *113*: 1207-1212, 1973.

Serial No. NIAMDD/CEB 7c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Action of Thyroid Hormones

Previous Serial Number: Same

Principal Investigators: J. Robbins, M.D., H. Carlson, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

Inactive

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 8c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Protein Synthesis in the Thyroid Gland

Previous Serial Number: Same

Principal Investigators: J. Robbins, M.D., F. Monaco, M.D.

Others: M. Brackbill

Cooperating Units: None

Man Years:

Total: 1 1/2
Professional: 1/4
Other: 1 1/4

Project Description:

The abnormality of thyroglobulin secretion in thyroid transplantable tumor 1-1c has been examined further. As previously described, there is a failure to incorporate the label from ^{3}H-ManNAc, a specific precursor of sialic acid, into thyroglobulin. All other components of the oligosaccharide chains were incorporated normally. The defect was shown to be due to a virtual absence of sialyl transferase specific for thyroglobulin in the cell-particle fraction of the tumor. Since this tumor also exhibits a failure of thyroglobulin release from cell membranes, these findings raise the possibility that sialic acid insertion in the terminal position of one of these two types of oligosaccharide chains of thyroglobulin is required for secretion of the protein from the cell into the colloid space.

These studies also provide some information about the specificity of sialyl transferases. The particulate fraction of the thyroid tumor cells had sialyl transferase activity capable of converting CMP-sialic acid into oligosaccharide-bound sialic acid of desialyzed fetuin and orosomucoid in almost normal amount despite the marked decrease in thyroglobulin-specific activity. The soluble fraction of the tumor and of normal thyroid contained sialyl transferase activity for all three receptors, but the thyroglobulin-specific activity was much lower than in normal thyroid particulate fractions.

Honors and Awards: None

Publications:

1. Monaco, F. and Robbins, J.: Membrane-Bound Thyroglobulin in an Experimental Thyroid Tumor of Rats. Biochem. Biophys. Acta 279: 118, 1972.

2. Monaco, F. and Robbins, J.: Incorporation of N-Acetyl-^{3}H Mannosamine and N-Acetyl-^{3}H Glucosamine into Thyroglobulin in Rat Thyroid In Vitro. J. Biol. Chem. 248: 2072, 1973.

3. Monaco, F. and Robbins, J.: Defective Thyroglobulin Synthesis in an Experimental Rat Thyroid Tumor: Lack of Membrane-Bound Sialyl Transferase Activity. J. Biol. Chem. 248: 2328, 1973.

Serial No. NIAMDD/CEB 9c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies in Thyroid Diseases

Previous Serial Number: CEB 19c

Principal Investigators: J. Robbins, M.D., H. Carlson, M.D., R. Temple, M.D.

Other Investigators: D. Murphy, M.D.

Cooperating Units: Behavioral Research, NIMH

Man Years:

Total: 1/4
Professional: 1/4
Other: 0

Project Description:

In further study of the effect of lithium ion on thyroid function and its role in the treatment of hyperthyroidism, we investigated thyroxine disappearance from blood. An effect of lithium on this parameter was suggested by our previous observation that the kinetics of iodine metabolism in some patients receiving lithium could not be explained solely by the effect on thyroid iodine secretion. Direct studies revealed that lithium caused a decrease in thyroxine disappearance from blood in hyperthyroid patients but not in euthyroid subjects.

A group of manic depressive patients receiving lithium therapy were also studied by the double isotope method using $[^{131}I]$iodide and $[^{125}I]$-thyroxine. The isotope ratio in urine reveals short-term fluctuations in thyroid iodine release, and a diurnal variation in this release is observed only by this method. In some of the patients studied, lithium caused a decrease in the diurnal rhythm of iodine release as well as a decreased rate of release, but the effect was transient, lasting only a few days. In one patient, a striking increase in iodine release followed cessation of lithium administration. It was of interest that manic depressive patients exhibited a diurnal rhythm of iodine release typical of normal subjects.

Honors and Awards: None

Publications:

1. Carlson, H. E., Temple, R. and Robbins, J.: Effect of Lithium on Thyroxine Disappearance in Man. J. Clin. Endocrin. Metab., in press.

Serial No. NIAMDD/CEB 10c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Ectopic Protein Production By Tumors

Previous Serial Number: CEB 9c

Principal Investigators: S. W. Rosen, Ph.D., M.D., B. D. Weintraub, M.D.

Other Investigators: A. S. Rabson, M.D., A. Tashjian, Jr., M.D., H. Sussman, M.D., F. Muggia, M.D., H. Hansen, M.D., I. Stotler, B.S., G. Krauth, M.S., Y. Kadesky, A.B.

Cooperating Units: Pathologic Anatomy Dept., Clinical Center; Harvard School of Dental Medicine, Boston, Mass.; Pathology Dept., Stanford University Medical Center, Palo Alto, Calif., NCI Oncology Unit, V.A. Hospital, Washington, D.C.

Man Years:

Total: 3 1/2
Professional: 1 1/4
Other: 2 1/4

Project Description:

I. Placental Protein "Markers"

A) In Vivo Studies

Serum and tumor extracts from a 75-year-old man with mixed epidermoid-adenocarcinoma of the pancreas contained the beta subunit of hCG (hCG-β) identified in three different radioimmunoassays and by gel chromatography. There was no evidence for either complete hCG or an alpha subunit, a situation somewhat analogous to isolated production of an immunoglobulin chain by certain lymphoid-plasma cell neoplasms. The question of possible heterogeneity of this ectopic hCG-β is being studied.

Serum from a 27-year-old woman with metastatic gastric carcinoid had 20,000 ng/ml of an alpha subunit identified by radioimmunoassay and by gel chromatography; this material was also present in tumor extracts. Again, there was no evidence for any other subunit nor for remotely comparable amounts of a complete glycoprotein trophic hormone. The substantial tumor concentration of this ectopic subunit may make it possible for us to isolate sufficient material for chemical characterization.

Both the alpha subunit and complete biologically active hCG were detected in the serum of still another cancer patient (47-year-old man with bronchogenic carcinoma), and tumor tissue from this patient has been successfully cultured in vitro (see below). If metabolic clearance rate data for the subunits in man is comparable to such data in rodents, in vivo tumor release of the alpha subunit exceeded its release of complete hCG, and is well reflected by the *in vitro* data for one of the clones (see below).

B. In Vitro Studies

We have recently studied a case of bronchogenic carcinoma associated with extopic production of biologically active hCG *in vivo*. A metastatic tumor nodule removed from this patient incorporated radioactive amino acids into hCG *in vitro* as demonstrated by short-term explant studies. The same tumor was used to establish uncloned cell lines in tissue culture, which were shown to secrete biologically active hCG into the medium. These uncloned cell lines have been maintained in serial culture for over three years and have continued to produce hCG. Recently, it has been demonstrated that these cells also secrete hCG-β as well as relatively large amounts of hCG-α.

In order to better define the origin of hCG and its subunits from these cells, we have established three clonal strains, which have been maintained in serial culture for over two years. Although each of the three clones secretes complete hCG, each secretes much larger amounts of either hCG-α or hCG-β, and the ratio of the two subunits produced varies greatly among the clones. Currently, studies are in progress to determine the mechanism of unbalanced subunit production in these cells.

II. Apparent Cooperativity in Radioimmunoassay

We have found that selected antisera to hormones display enhanced binding of labeled hormone in the presence of small amounts of unlabeled hormone. Various binding analyses have shown that this phenomenon is analogous to other known cooperative protein-ligand interactions. This is supported by studies of antibody fragments which show retention of enhanced binding of divalent $F(ab)_2$ fragments but loss of this effect in univalent Fab fragments. Studies are in progress to further define this phenomenon and to further explore its usefullness in radioimmunoassay.

Honors and Awards: None

Publications:

1. Rabson, A. S., Rosen, S. W., Tashjian, A. H. and Weintraub, B. D.: Production of Human Chorionic Gonadotropin *In Vitro* by a Cell Line Derived From a Carcinoma of the Lung. *J. Nat. Cancer Inst.* *50*: 669, 1973.

2. Tashjian, A. H., Jr., Weintraub, B. D., Barowsky, N. J., Rabson, A. S. and Rosen, S. W.: Subunits of Human Chorionic Gonadotropin: Unbalanced Synthesis and Secretion by Clonal Cell Strains Derived From a Bronchogenic Carcinoma. Proc. Nat. Acad. Sci. USA, in press.

3. Weintraub, B. D., Rosen, S. W., McCammon, J. A. and Perlman, R. J.: Apparent Cooperativity in Radioimmunoassay of Human Chorionic Gonadotropin. Endocrinology 32: 1250, 1973.

4. Charles, M. A., Claypool, R., Schaaf, M., Rosen, S. W. and Weintraub, B. D.: Lung Carcinoma Associated With Ectopic Production of Three Placental Proteins: Human Chorionic Gonadotropin, Human Chorionic Somatomammotropin and Placental Alkaline Phosphatase. A Case Study. Arch. Intern. Med., in press.

Serial No. NIAMDD/CEB 11c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Macro LDHemia

Previous Serial Number: None

Principal Investigator: S. W. Rosen, Ph.D., M.D.

Other Investigators: C. R. Kahn, M.D., R. Temple, M.D., D. Thomas, M.D., N. Papadopoulos, M.D., E. Vesell, M.D.

Cooperating Units: Clinical Pathology Dept., Clinical Center; Walter Reed Army Institute of Research; Dept. of Pharmacology, Hershey Medical Center, Hershey, PA.

Man Years:

Total: 1/4
Professional: 1/4
Other: 0

Project Description:

A sixty-four year old woman has been investigated for increased serum lactate dehydrogenase (LDH) activity present for at least two years. Usual causes of increased serum LDH activity were excluded. Gel chromatography demonstrated that 99 percent of serum LDH activity was present in a form larger than normal; the remaining one percent of LDH activity was present in normal form. LDH activity in saliva and duodenal fluid was present entirely in a normal form. Serum protein studies demonstrated an increase in immunoglobulin A concentration, predominantly of the kappa light chain type. Immunoelectrophoretic and immunoprecipitation studies showed that the vast majority of LDH activity was associated with this paraprotein. LDH isoenzyme separation revealed an apparent additional band between the normal third and fourth isoenzyme bands corresponding to the region of migration of the immunoglobulin A paraprotein. This macromolecular form of LDH bound to immunoglobulin A is probably cleared from the circulation less rapidly than normal LDH producing a persistently high serum LDH activity.

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 12c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies With Luteinizing Hormone-Releasing Factor (LRF)

Previous Serial Number: None

Principal Investigator: S. W. Rosen, Ph.D., M.D.

Other Investigators: A. Rogol, M.D., J. Rochefort, Ph.D.

Cooperating Units: Ayerst Laboratories, New York, New York

Man Years:

Total: 1/4
Professional: 1/4
Other: 0

Project Description:

Synthetic decapeptide LRF was obtained from Ayerst and is being administered subcutaneously to normal volunteers and to patients with a variety of pituitary, hypothalamic and gonadal disorders. Serial measurements are being made of serum radioimmunoassayable FSH and LH.

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 13c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies With Thyrotropin Releasing Hormone (TRH)

Previous Serial Number: 10c

Principal Investigator: S. W. Rosen, Ph.D., M.D.

Other Investigator: R. Sachson, M.D.

Cooperating Units: None

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

Inactive

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 14c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: TSH and Prolactin Studies

Previous Serial Number: None

Principal Investigators: B. D. Weintraub, M.D. (A-D), S. W. Rosen, Ph.D., M.D. (E-F)

Other Investigators: I. Kourides, M.D., F. Maloof, M.D., K. Gautvik, M.D., A. Tashjian, Jr., M.D. (A-D), A. Rogol, M.D., R. Dimond, M.D. (E-F)

Cooperating Units: Walter Reed Army Institute of Research; Harvard Medical School, Boston, Mass.

Man Years:

Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

A) Response of Prolactin and TSH to Suckling

Our studies in postpartum women have shown that suckling consistently produces a large increment in serum prolactin but produces no rise in serum TSH. Moreover, when these same postpartum women are given exogenous TRH in various doses, there is always a parallel response in both TSH and prolactin at all doses. The latter data suggest that the lack of TSH response during suckling was not simply due to relative insensitivity of thyrotrophs to small amounts of endogenous TRH. These studies indicate that TRH is probably not the physiologic mediator of the suckling response.

B) Development of Radioimmunoassay to hTSH-α and hTSH-β

We have prepared hTHS-α and hTSH-β by dissociating hTSH in 1 M proprionic acid and separating the subunits by gel and ion exchange chromatography. Purified subunits were used to induce antibodies and were labeled with ^{125}I, providing the necessary reagents for subunit radioimmunoassays. These assays were highly specific for the respective subunit and permitted determination of free subunit concentrations in unfractionated serum. We have detected hTSH-β in primary myxedema serum and have found that the

G) Radioimmunoassay of Prolactin: Some Methodologic Problems

Radioiodination of both bovine (bPRL) and human (hPRL) prolactin by the conventional chloramine-T method gave structurally modified labeled prolactins as evidenced by their greater exclusion from Sephadex G100, decreased stability, and decreased affinity for anti-PRL relative to prolactins radioiodinated with lactoperoxidase. The apparent M.W. of the latter on G100 was in excellent agreement with the known M.W. of human and bovine PRL.

Honors and Awards: None

Publications:

1. Sachson, R., Rosen, S. W., Cuatrecasas, P., Roth, J. and Frantz, A. G.: Prolactin Stimulation by Thyrotopin-Releasing Hormone in a Patient With Isolated Thyrotropin Deficiency. New Eng. J. Med. 287: 972-973, 1972.

2. Bode, H. H., Danon, M., Weintraub, B. D., Maloof, F. and Crawford, J. D.: Partial Target Organ Resistance to the Action of Thyroid Hormone. J. Clin. Invest. 52: 776, 1973.

3. Gautvik, K. M., Weintraub, B. D., Graefer, C. T., Maloof, F., Zuckerman, J. E. and Tashjian, A. H., Jr.: Serum Prolactin and TSH: Effects of Nursing and Pyro Glu-His-Pro NH_2 Administration in Postpartum Women. J. Clin. Endocrinol., in press.

Serial No. NIAMDD/CEB 15c
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Interaction of Insulin with Receptors in Liver

Previous Serial Number: 11c

Principal Investigators: C. R. Kahn, M.D., A. Soll, M.D., I. D. Goldfine, M.D.
P. Gorden, M.D., J. Roth, M.D.

Other Investigators: D. M. Neville, Jr., M.D., R. W. Bates, M.D.

Cooperating Units: Laboratory of Neurochemistry, NIMH; Laboratory of Nutrition and Endocrinology, NIAMDD

Man Years:

Total: 3 1/2
Professional: 2 1/2
Other: 1

Project Description:

The interaction of insulin with its receptor in plasma membranes of rat liver has been shown to be more complex than originally believed. The insulin receptor population is not homogeneous but is composed of at least two types of sites: a high affinity-low capacity and low affinity-high capacity site. The apparent equilibrium constant for the high affinity site is about 2×10^9. Both the affinity constants and the number of receptor sites are influenced by temperature and ionic strength. In addition to the binding site, the purified plasma membranes of liver appear to possess a degradation site for insulin. The reaction is relatively "specific" for the insulin structure. The K_m for the "insulin specific enzyme" is about 1.7×10^{-7} M.

The role of the insulin receptor in insulin resistance is currently under study. In several forms of genetic and acquired obesity, a deficiency of insulin receptors is found which correlates with the insulin resistance observed. Diet restriction causes an increase in the number of receptors and an improvement in insulin sensitivity. Maintenance of high levels of insulin during the time of fasting prevents the increase in insulin binding sites.

In the insulin resistance of growth hormone and glucocorticoid excess there is also a decrease in insulin binding to the liver plasma membranes. This can be demonstrated by exogenous administration of these hormones or by implantation of the MtT pituitary tumor which secretes ACTH, growth hormone, and prolactin. Adrenalectomy of the tumor-bearing animals causes the insulin binding to return toward normal.

Finally, the insulin-receptor interaction is being used as a radioreceptor assay for insulin-like hormones. The acid-ethanol soluble nonsuppressible insulin-like activity from plasma (NSILA-S) demonstrates potency in the receptor assay parallel to its bioactivity. Bovine diabetogenic peptide, which also has insulin-like effects *in vitro*, has no activity toward the receptor.

Honors and Awards: None

Publications:

1. Freychet, P., Kahn, C. R., Roth, J. and Neville, D. M., Jr.: Insulin Interactions with Liver Plasma Membranes: Independence of Binding of the Hormone and Its Degradation. *J. Biol. Chem.* *247*: 3953-3961, 1972.

2. Kahn, C. R., Neville, D. M., Jr., Gorden, P., Freychet, P. and Roth, J.: Insulin Receptor Defect in Insulin Resistance: Studies in the Obese Hyperglycemic Mouse. *Biophys. Biochem. Res. Commun.* *48*: 135-142, 1972.

3. Freychet, P., Laudat, M. H., Laudat, P., Rosselin, G., Kahn, C. R., Gorden, P. and Roth, J.: Impairment of Insulin Binding to the Fat Cell Membrane in the Obese Hyperglycemic Mouse. *FEBS Letters* *25*: 339-342, 1972.

4. Kahn, C. R., Neville, D. M., Jr., and Roth, J.: Insulin-Receptor Interactions in the Obese-Hyperglycemic Mouse: A Model for Insulin Resistance. *J. Biol. Chem.* *248*: 244-250, 1973.

Serial No. NIAMDD/CEB 16c
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Factors Affecting Insulin Receptor Concentrations

Previous Serial Number: 12c

Principal Investigators: I. D. Goldfine, M.D., J. Roth, M.D., C. R. Kahn, M.D.

Other Investigators: D. M. Neville, Jr., M.D., R. W. Bates, M.D.

Cooperating Units: Laboratory of Neurochemistry, NIMH; Laboratory of Nutrition and Endocrinology, NIAMDD

Man Years:

Total: 3
Professional: 2
Other: 1

Project Description:

Studies in animals and man have shown that sensitivity to exogenous and endogenous insulin is altered by hormones and various metabolic states. In thymocytes from rats and mice we demonstrated that insulin binding correlated with insulin stimulated α aminoisobutyric (AIB) acid influx. In thymocytes from the insulin resistant obese hyperglycemic mouse, insulin had small effects on AIB influx which correlated with a severe decrease in insulin receptor concentrations. In cells from insulin sensitive hypophysectomized rats there was an increase in insulin stimulated AIB influx. Administration of growth hormone to hypophysectomized rats did not change insulin receptor binding.

Insulin receptor binding was decreased in liver membranes from rats bearing a pituitary tumor secreting two insulin antagonists, ACTH and growth hormone. In contrast glucagon receptor binding was unchanged. Administration of ACTH and growth hormone alone to normal rats also decreased insulin receptor binding. Hypophysectomy and adrenalectomy had minor effects.

It was concluded that changes in insulin receptor binding are important factors in states of altered sensitivity to insuliin.

Honors and Awards: None

Publications:

1. Goldfine, I. D., Gardner, J. D. and Neville, J. D.: Insulin Action in Rat Thymocytes: I. Binding of ^{125}I Insulin and Stimulation of α Aminoisobutyric Acid Transport. J. Biol. Chem. 247: 6919-6926, 1972.

2. Goldfine, I. D. and Sherline, P.: Insulin Action in Rat Thymocytes: II. Independence of Insulin and Cyclic AMP. J. Biol. Chem. 247: 6927-6931, 1972.

Serial No. NIAMDD/CEB 17c
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Human Growth Hormone (HGH) in Man

Previous Serial Number: 13c

Principal Investigators: P. Gorden, M.D., C. R. Kahn, M.D., A. J. Sober, M.D., J. Roth, M.D.

Other Investigators: C. Hendricks

Cooperating Units: None

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description: Inactive

Serial No. NIAMDD/CEB 18c
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Nature of Plasma Insulin in Man

Previous Serial Number: 14c

Principal Investigators: P. Gorden, M.D., C. R. Kahn, M.D., J. Roth, M.D.

Other Investigators: C. Hendricks

Cooperating Units: None

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

Inactive

Serial No. NIAMDD/CEB 19c
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Interaction of Polypeptide Hormones with Their Receptors in Target Tissues

Previous Serial Number: 15c

Principal Investigators: J. R. Gavin, III, Ph.D., M. Lesniak, J. Archer, M.D., P. Gorden, M.D., J. Roth, M.D., W. McGuffin, M.D.

Other Investigators: C. Hendricks, D. Buell, M.D.

Cooperating Units: General Laboratories and Clinics, NCI

Man Years:

Total: 5
Professional: 3 1/2
Other: 1 1/2

Project Description:

Since polypeptide hormones exert their effects on specific cell receptors, the accessibility of the circulating cell makes this tissue desirable for studies of hormone interactions in man. Using biologically-active monoiodoinsulin, we find that circulating and cultured human lymphocytes have receptors for insulin that have the same affinity and biologic specificity for insulin as the receptors studied in liver and fat cells of rats.

Peripheral lymphocytes have been used to study directly insulin-receptor interactions in normal individuals and in patients with conditions characterized by alterations in insulin sensitivity. To study insulin binding, lymphocytes were incubated with ^{125}I-insulin at 0.1 ng/ml. The parameters measured were the maximal binding of ^{125}I-insulin per 70 x 10^6 cells (B_{max}) and the concentration of unlabeled hormone required for half-maximal inhibition of ^{125}I-insulin binding (ng/ml) to the cells (1/2 B_{max}).

Cells from obese hyperglycemic insulin resistant patients bind only about half as much of a tracer of ^{125}I-insulin (0.1 ng/ml) as cells of non-obese subjects and display a shallower curve of displacement on addition of physiological amounts of unlabeled insulin. In six obese patients, caloric restriction produced amelioration not only of the glucose intolerance and insulin resistance, but of the defective insulin binding as well.

The presence of high affinity sites for insulin on cultured human lymphocytes has provided a means for the development of a sensitive radioreceptor assay to measure plasma insulin components and substances with insulin-like activity in vitro. Endogenous plasma components from patients with obesity, diabetes, and islet cell tumor were partially purified and assayed. The insulin component has the same biologic specificity as pancreatic porcine insulin, whereas the proinsulin-like component has biologic specificity similar to purified porcine proinsulin. Pancreatic proinsulin intermediates inhibited binding to the cultured lymphocyte in direct proportion to their biologic activity. A highly purified acid-ethanol soluble non-suppressible insulin-like activity from plasma (NSILA-S) demonstrated the same potency in the receptor assay as in the bioassay while it was only 1% as active in the immunoassay. Bovine diabetogenic peptide (BDP), which also has insulin-like effects in vitro, had no activity in the lymphocyte radioreceptor assay.

Specific water-soluble insulin receptors are released from cultured human lymphocytes when incubated in phosphate-buffered saline at cell concentrations of $> 2 \times 10^7$ cells/ml at 30° or 37°C. Cells from high density/stationary phase cultures release receptors more readily than exponentially growing cells, although they have identical numbers of receptors/cell. The receptors isolated in this manner retain the specificity and sensitivity observed in intact cells.

Specific growth hormone receptors have been characterized in cultured human lymphocytes. Cell line #IM-9 contains approximately 4000 binding sites with an apparent affinity constant of 4×10^8 L/M. These receptors have been used to develop a specific, sensitive radioreceptor assay for plasma growth hormone components. With this method, the "little" growth hormone component which has been isolated by filtration of plasma on Sephadex G-100 has biologic specificity identical to purified pituitary hGH whereas the "big" growth hormone component is only about 20% as potent.

Honors and Awards: None

Publications:

1. Gavin, J. R., III, Buell, D. N. and Roth, J.: Water-Soluble Insulin Receptors from Human Lymphocytes. Science 178: 168-170, 1972.

2. Gavin, J. R., III, Mann, D. L., Buell, D. N. and Roth, J.: Preparation of Solubilized Insulin Receptors from Human Lymphocytes. Biochem. Biophys. Res. Comm. 49: 870-876, 1972.

3. Lesniak, M. A., Roth, J., Gorden, P. and Gavin, J. R., III: Application of Growth Hormone Receptors in Cultured Human Lymphocytes to Radioreceptor Assay. Nature New Biol. 241: 20-22, 1973.

4. Gavin, J. R., III, Gorden, P., Roth, J., Lesniak, M. A. and Archer, J. A.: Characteristics of the Human Lymphocyte Insulin Receptor. J. Biol. Chem. 248: 2202-2207, 1973.

5. Archer, J. A., Gorden, P., Gavin, J. R., III, Lesniak, M. A. and Roth, J.: Insulin Receptors in Human Circulating Lymphocytes: Application to Studies of Endocrine Disorders in Man. J. Clin. Endo. Metab. 36: 627-631, 1973.

6. Gorden, P., Gavin, J. R., III, Kahn, C. R., Archer, J. A., Lesniak, M., Hendricks, C., Neville, D. M., Jr., and Roth, J.: Application of Radioreceptor Assay to Circulating Insulin, Growth Hormone, and to Their Tissue Receptors in Animals and Man. Pharmacol. Rev., in press.

7. Roth, J.: Peptide Hormones Binding to Receptors: A Review of Direct Studies in vitro. Metabolism, in press.

Serial No. NIAMDD/CEB 20c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mechanism of Thyroid Hormone Action

Previous Serial Number: 16c

Principal Investigators: M. McClung, M.D., J. E. Rall, M.D., Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Other: 1

Project Description:

It has been shown by many groups of investigators that the regulation of cellular biochemical mechanisms by several of the polypeptide hormones involves cyclic AMP (cAMP) and cAMP-dependent protein kinases. Using cAMP dependent protein kinase from bovine skeletal muscle, the effect of L-triiodothyronine (T_3) on the regulation of protein kinase activity by cAMP has been studied. It was found that T_3 does not inhibit cAMP-independent kinase activity but that it does inhibit cAMP-dependent kinase activity using histone as a substrate. This effect is dependent upon thyroid hormone concentration. Inhibition was seen at $10^{-7}M$ T_3 and was 100% at $10^{-4}M$ T_3. In addition, T_3 decreases the binding of cAMP to protein kinase by 25% as determined by the Gilman assay method. Preliminary equilibrium dialysis studies have also demonstrated T_3 binding to cAMP dependent protein kinase. This suggests that the metabolic effects of thyroid hormone might, at least in part, be mediated through the modulation of cAMP-dependent protein kinase activity. The catalytic and regulatory subunits of protein kinase have been separated and purified for further kinetic studies.

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 21c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Thyroid Iodoproteins

Previous Serial Number: 17c

Principal Investigators: J. Bilstad, M.D.

Other Investigators: J. E. Rall, M.D., Ph.D., G. Salvatore, M.D., H. Edelhoch, Ph.D.

Cooperating Units: Office of the Director, NIAMDD; Istituto di Patologia Generale, Università di Napoli, Naples, Italy

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

The goal of this project is to determine the number, size and chemical composition of the primary polypeptide chains of the thyroid iodoproteins, 19 S thyroglobulin (mw 660,000) and the 27 S iodoprotein (mw ~1,300,000).

In the presence of dissociating agents normally iodinated 19 S partially dissociates into a 12 S species (mw 330,000), the smallest non-covalently bound subunit. Following complete reduction and aklylation considerable size heterogeneity was noted. When analyzed by gel electrophoresis in the presence of the detergent, SDS, 12 S was still the predominant band. In addition there are approximately 10 bands with molecular weights between 10,000 and 300,000. Attempts to find dissociating conditions which would result in greater dissociation have generally been unsuccessful. Various methods of alkylating the reduced SH groups have been used, including trimethylaminoethylation with bromocholine bromide, but these also have not been helpful. The possibility of proteolysis as an explanation for the production of the multiple bands is also being explored further.

Seventy percent formic acid was found previously to cause a time dependent disappearance of the heavier components with the appearance of a major component with a mw of 45,000. This species was isolated by gel filtration, and N-terminal analysis by Edman degradation revealed no N-terminal residues, possibly due to formylation. Other organic acids were tested and acetic acid gave similar results. This 45,000 mw component produced by the acetic acid treatment is currently being isolated and further characterized.

Attempts have been made to isolate fucose containing components of reduced and alkylated thyroglobulin by affinity chromatography with a fucose-binding protein covalently linked to agarose. Preliminary results suggest that the complex band pattern seen in SDS gel electrophoresis with the reduced and alkylated thyroglobulin prior to affinity chromatography remains in the material which is not bound and in that which is bound and eluted with dissociating agents. Analysis of these fractions for fucose content is now underway.

Biosynthesis of the 27 S iodoproteins was also studied *in vivo* in rats to determine its relationship to 19 S. Evidence was obtained that 19 S is the immediate precursor of 27 S. Formation of 27 S was found to be independent of *de novo* protein synthesis but dependent on iodination. It appears that above a certain threshold iodine level, 27 S is formed by chemical alterations produced as a by-product of iodination of the tyrosyl residues, probably involving oxidative side-reactions.

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 22c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Antimitotic Agents and Adrenal Secretion

Previous Serial Number: 18c

Principal Investigators: J. Wolff, M.D., Ph.D., R. Temple, M.D.

Other Investigators: G. H. Cook

Cooperating Units: None

Man Years:

Total: 1 1/4
Professional: 1/2
Other: 3/4

Project Description:

Studies on the presumptive role of microtubules in steroid secretion by Y-1 adrenal tumor cells has continued. The antimicrotubular agents colchicine, vinblastine or podophyllotoxin have been found to stimulate steroid production by Y-1 adrenal tumor cells in culture. The amounts of steroid secreted are comparable to those produced by maximal levels of ACTH and the products, 20 α-dihydroprogesterone and 11β-hydroxy-20α-dihydroprogesterone, are identical. ACTH and vinblastine stimulation is not additive. As with ACTH, stimulation occurs between the cholesterol and Δ4-pregnenolone steps, and it is inhibited by aminogluthethimide and cycloheximide. It does not, however, involve activation of the adenylate cyclase system. The time course of stimulation differs from ACTH in that the antimicrotubular agents have a 6-9 hr lag period which is absent with ACTH. D_2O, an agent which stabilizes microtubules, inhibits stimulation of steroid secretion by vinblastine, ACTH or cAMP. The precise role of protein synthesis in steroid secretion is being studied by means of these drugs.

Steroid secretion by Leydig tumor cells in culture is also stimulated by vinblastine with a lag period but this is less extensive than with cAMP. This stimulation is also sensitive to D_2O.

We propose that the antimicrotubular agents facilitate access of cholesterol to the mitochondrion and that this may also be true for hormonally-stimulated steroid secretion.

Honors and Awards: None

Publications:

1. Temple, R. and Wolff, J.: Stimulation of Steroid Secretion by Antimicrotubular Agents. J. Biol. Chem. 248: 2691-2698, 1973.

Serial No. NIAMDD/CEB 23c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Thyroid Plasma Membranes

Previous Serial Number: 20c

Principal Investigators: J. Wolff, M.D., Ph.D., W. V. Moore, M.D., Ph.D.

Other Investigators: G. H. Cook

Cooperating Units: None

Man Years:

Total: 2
Professional: 1 1/4
Other: 3/4

Project Description:

The nature of the beef thyroid membrane adenylate cyclase has been further investigated. The effect of purine and pyrimidine nucleotides on the activation of adenylate cyclase by thyrotropin and prostaglandin on E_1 in beef thyroid plasma membranes was investigated. At 1 mM ATP concentration, GTP increases basal and thyrotropin-stimulated activity 2- to 4-fold at concentration of 1 to 30 μM. Significant stimulation occurs at 0.1 μM. Greater maximal stimulation occurs with ITP (5- to 7-fold) over the concentration range of 20 to 40 μM. In order of decreasing maximal effect the nucleoside triphosphates are ITP > dGTP > GTP > XTP. At low concentrations GTP > ITP. Pyrimidine nucleotides had low activity. The enhancement occurs also with IDP and GDP, and at 1 mM with 5'-IMP and 5'-GMP. The ITP and IDP effects have a 1 to 2 min lag period before they become manifest.

F^--activated cyclase is not stimulated and is partially inhibited at high nucleotide concentrations; thus, thyrotropin-stimulated cyclase may exceed F^--stimualted activity in some conditions.

Prostaglandin E_1-stimulated activity is also enhanced by these nucleotides but, in contrast to thyrotropin activity, maximal stimulation by GTP ≃ dGTP ≃ ITP > XTP. Potassium ion increases thyrotropin-stimulated adenylate cyclase in the presence of 20 μM ITP, whereas there is no K^+ effect on stimulation evoked by prostaglandin E_1 even in the presence of

ITP. Furthermore, the prostaglandin and thyrotropin stimulations are additive in the absence and in the presence of ITP and GTP.

It is concluded that the nucleotides interact with the cyclase system not at the catalytic site, that the nucleotides can interact at more than one site, and that the thyrotropin and prostaglandin receptors are probably separate.

In addition, the binding of tritiated prostaglandin E_1 [^{3}H-PGE$_1$] to beef thyroid membranes was investigated by a micro-discontinuous gradient centrifugation method. Two populations of binding sites were found. Binding at pH 7 was found to require Ca^{++} (half-maximal concentration 1 mM), was heat labile, was inhibited by prostaglandin E_2 and $F_{1\alpha}$, arachidonic acid and 7-oxa-13-prostynoic acid, and was readily reversible. With 5 mM Ca^{++} at pH 7.0, the affinity constant was $2.6 \times 10^8 M^{-1}$. ITP, GTP and dGTP displaced bound ^{3}H-PGE$_1$ from the membranes at nucleotide concentrations that have been shown to enhancnce PGE$_1$ activation of adenylate cyclase.

The other population of sites was heat stable, had a pH optimum of 3.6 and a pK of 5.1. Calcium ion, although not required, enhanced the binding of ^{3}H-PGE$_1$ and shifted the optimum pH to 4.5-4.7; there were two binding components with apparent affinity constants of $1.6 \times 10^{10} M^{-1}$ and $1.6 \times 10^8 M^{-1}$. These sites had a much lower order of chemical specificity, being less sensitive to prostaglandin analogues or antagonists and insensitive to ITP, GTP and thyrotropin.

It is concluded that binding of ^{3}H-PGE$_1$ at pH 7 fulfills the requirements of a physiologic binding site whereas binding at pH 5 is non-specific.

The role of PGE synthesis has also been studied with indomethacin. Indomethacin, in concentrations up to 400 μM, fails to alter the stimulation by thyrotropin in various thyroid preparations of [1-^{14}C]glucose oxidation, hormone and iodine secretion, and adenylate cyclase activity. It is concluded that prostaglandin synthesis is not an obligatory step in the activation of these thyroid functions by thyrotropin.

Honors and Awards: None

Publications:

1. Covelli, I., Frati, L., and Wolff, J.: Carboxymethylation of the Histidyl Residues of Insulin. Biochemistry 12: 1043-1047, 1973.

2. Wolff, J. and Moore, W. V.: The Effect of Indomethacin on the Response of Thyroid Tissue to TSH. Biochem. Biophys. Res. Comm. 51: 34-39, 1973.

3. Wolff, J. and Cook, G. H.: Activation of Thyroid Membrane Adenylate Cyclase by Purine Nucleotides. J. Biol. Chem. 248: 350-355, 1973.

Serial No. NIAMDD/CEB 24c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Thyroid Hormone Secretion

Previous Serial Number: 21c

Principal Investigators: J. Wolff, M.D., Ph.D., B. Bhattacharyya, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1 1/4
Professional: 1 1/4
Other: 0

Project Description:

The inhibition of TSH-stimulated thyroid secretion by vinblastine and colchicine has led us to propose the hypothesis that the secretion of preexisting dimeric tubulin subunits. Preliminary electronmicroscopic findings suggest an increase in microtubules/unit area shortly after TSH stimulation but the statistical problems are formidable.

Studies of the relation of subunit synthesis to secretion suggest that, although TSH stimulates synthesis of tubulin dimers, this occurs much too late to account for the secretory response.

Attempts to characterize tubulin from thyroid tissue have been slowed by the extreme lability of this protein in crude thyroid preparations. Rapid separation methods have succeeded in yielding an almost pure protein and further characterization is now in progress. So far, the protein is like the brain protein, with subunit molecular weight of ~57,000.

Honors and Awards: None

Publications:

1. Williams, J. A. and Wolff, J.: Colchicine-Binding Protein and the Secretion of Thyroid Hormone. J. Cell Biol. 54: 157-165, 1972.

2. Temple, R., Berman, M., Robbins, J. and Wolff, J.: The Use of Lithium in the Treatment of Thyrotoxicosis. *J. Clin. Invest.* *51*: 2746-2756, 1972.

3. Temple, R., Berman, M., Carlson, H. E., Robbins, J. and Wolff, J.: The Use of Lithium in Graves' Disease. *Mayo Clin. Proc.* *47*: 872-878, 1972.

Serial No. NIAMDD/CEB 25c
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effect of Insulin on DNA Synthesis in Cultured Human Fibroblasts

Previous Serial Number: None

Principal Investigators: M. M. Rechler, M.D.

Other Investigators: I. D. Goldfine, M.D., C. A. Wells, Ph.D., J. Podskalny

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Insulin stimulates DNA synthesis in human fibroblasts in tissue culture. This is reflected in a net synthesis of DNA (determined chemically) and by an increased rate of incorporation of ^{3}H-thymidine into DNA. Thymidine incorporation is stimulated by as little as 100 ng/ml of insulin, and the maximum effect is seen at $\geqq$ 10 μg/ml. The stimulation is first seen 8-10 hours after insulin is added, and is maximal at 16 hours. Typically, insulin-treated fibroblasts incorporate 2-3 times as much ^{3}H-thymidine into DNA as do control cells. Insulin appears to stimulate more cells to make DNA (determined by autoradiography). Stimulation of DNA synthesis by fetal calf serum and by insulin proceed by different mechanisms since the two effects are additive at optimal concentrations of each component. The insulin effect on DNA synthesis is discrete and specific, since uridine incorporation into RNA is not stimulated and only a low level stimulation of leucine incorporation into protein is seen. These results suggest that insulin may have a significant role in promoting cell growth.

Honors and Awards: None

Publications: None

Investigations in the past year have concentrated on cystic fibrosis of the pancreas (CF), other genetic pancreatic disorders, and on the respiratory distress syndrome (RDS).

A. CYSTIC FIBROSIS OF THE PANCREAS (CF)

I. Biochemical and Metabolic Studies

a) Tissue culture studies

Methylation of transfer RNA (tRNA) and ribosomal RNA (rRNA) *in vivo* and *in vitro* by fibroblasts from normal humans and patients with cystic fibrosis: Rennert et al. (Pediatrics 50:485, 1972; Clin. Pediat. 11:351, 1972) have reported that fibroblasts in tissue culture from patients with cystic fibrosis show decreased methylation of total RNA. In addition, these investigators have observed that fibroblasts from obligate heterozygotes for CF also manifest reduced RNA methylation to a figure intermediate between controls and CF homozygotes. Such an alteration, if consistently demonstrable, would be of major importance in several respects.

In this study control and CF fibroblasts were cultured in the presence of L-(methyl-^{14}C) methionine and 5-^{3}H uridine. RNA then was extracted and fractionated by sodium dodecyl sulfate sucrose density gradient centrifugation. The ratios of ^{14}C/^{3}H incorporation revealed that tRNA, 18S rRNA, and 28S rRNA of CF cells were methylated to at least as great an extent as in the control cells. In addition, the methylated base composition of human fibroblast tRNA and rRNA was identified for the first time by two dimensional chromatography. The observed bases were the same for CF and control cells and identical to the pattern previously found for RNA from yeast, various animal tissues and HeLa cells.

Methylation rates were also measured *in vitro* by incubating fibroblast extracts with *E. coli* tRNA and S-adenosyl (methyl-^{3}H) methionine. It was found that the activities of CF extracts were equal to or greater than those of control fibroblasts and the patterns of methylated bases were again identical. Therefore, CF fibroblasts show normal methylation of tRNA and rRNA *in vivo* and no deficiency in tRNA methylases.

A possible explanation for the differences in the results presented in this study and those of Rennert might involve the different growth conditions used by these two laboratories in labeling cells. (Dr. P. Farrell, Dr. M. Klagsbrun, NIAMDD).

Lower protein contents of cystic fibrosis fibroblasts: Rate of growth in normal versus CF fibroblasts in tissue culture was studied by three methods: cell counts, DNA determinations, and the Lowry protein method. Five fibroblast lines from control subjects and five lines from CF patients (homozygotes) were studied.

Results showed comparable rates of cell proliferation of both control and CF cell lines. The DNA content per cell was comparable in both groups. However, the Lowry protein based on DNA levels was consistently lower in CF cell lines during the logarithmic growth phase ($p<0.01$) and only in the post-confluent phase reached levels comparable to those of control cells. The significance of these findings is being investigated further.

These results explain the previous observation in our laboratory of higher 3-O-methylglucose binding which was based on Lowry protein determinations. When corrected by these results, 3-O-methylglucose binding is equal in both cell types.

The significantly elevated glycogen levels in CF fibroblasts previously reported (Pallavicini et al., J. Pediatrics, 77:280, 1970), although expressed as a ratio to Lowry protein determinations, cannot be accounted for solely on this basis. Even with the correction, the levels of this polysaccharide are very significantly increased in the pathologic cells as compared to the fibroblasts from normal individuals. (Dr. J. Pallavicini, M. Ulane (guest worker), J. Podskalny (guest worker).

b) Genetic markers in cystic fibrosis

Although the biochemical defect in cystic fibrosis is unknown, several reports have appeared in recent years of possible genetic markers with promise for use in genetic counseling and hopefully for prenatal diagnosis. The most promising of these seemed to be a factor or factors present in serum of homo- and heterozygotes which produce dyskinesia of rabbit tracheal epithelial cilia, and a reported defect in RNA methylation in cultured fibroblasts and lymphocytes. Before investing in a full scale approach to these problems we visited the laboratories of several of the leaders in these investigations and also gave them a number of coded samples of either serum or viable lymphocytes for use in their own laboratories. The results of the blind studies are shown:

	CF Homozygotes		Control Subjects	
	True Pos.	False Neg.	True Neg.	False Pos.
Ciliary Dyskinesia				
Center A	5/20	15/20	10/18	8/18
Center B (incomplete)	14/17	3/17	1/8	7/8
RNA Methylation (lymphocytes)	3/12	9/12	5/10	5/10

Therefore, it appears that reports of heterozygote and antenatal diagnosis by *in vitro* tests are at least premature, as they are very unreliable in blind studies. (Dr. R. Wood, NIAMDD)

c) Effects of vitamin E deficiency in man

The biological manifestations of vitamin E deficiency are more protean in various animal species than for any other nutrient. However, man has not been shown conclusively to develop clinical symptoms of vitamin E deficiency. Patients with cystic fibrosis available to us represent the ideal experimental model of profound vitamin E deficiency, often present for a period of many years. Study of these subjects with sophisticated methods in a comprehensive approach will permit much better definition of the changes in physiology which must be produced by the absence of this ubiquitous vitamin as well as defining the necessity of supplements in the treatment of CF patients.

Although abnormal erythrocyte hemolysis *in vitro* in the presence of hydrogen peroxide has been observed in many, but not all, of the individuals with low plasma tocopherol, there is considerable disagreement on the question of whether significant hemolysis occurs *in vivo* with vitamin E deficiency. Excessive urinary excretion of creatine and occasional evidence of focal necrosis of striate and myocardial muscle had been observed occasionally in vitamin E deficient humans. Ceroid pigment accumulation occurs regularly in smooth muscle of vitamin E deficient humans. There is reason to believe from studies in two vitamin E deficient patients with a-beta-lipoproteinemia that ultrastructural abnormalities in the membranes of mitochondria and endoplasmic reticulum occur with dramatic improvement in these subcellular structures after supplementation with this vitamin.

It is, therefore, proposed to evaluate several biochemical and morphologic parameters of vitamin E deficiency in a group of 85 patients with CF and other pancreatic insufficiency states such as hereditary pancreatitis and Shwachman-Diamond syndrome. Previous studies of human vitamin E deficiency have been limited in scope and have been hampered by the small population of subjects available for study and by the lack of suitable controls. To obviate this difficulty, each patient will be used as his own control.

Preliminary results are as follows: Plasma α-tocopherol (α-T) determined after thin layer chromatography, was uniformly decreased in 42 CF patients with pancreatic achylia (mean = 125 μg/100 ml, range = 5-410; control mean = 730 μg/100 ml, range = 550-1060). Erythrocyte α-T was decreased in parallel fashion and averaged 42 μg/100 ml of packed red cells in 5 patients as compared with a normal mean of 230 ± 13 μg/100 ml (Bieri and Poukka, Internat. J. Vit. Res., 40:344, 1970). In general, the degree of reduced α-T appeared to correlate with the extent of steatorrhea. Oral supplementation with 3-5 I.U./kg/day of water-miscible α-T resulted in normal plasma levels except in the presence of marked, concomitant hepatic dysfunction where higher supplements were required. Furthermore, two supplemented patients at autopsy showed normal levels of tissue α-T, as given in the table below in comparison with the data on one deficient subject; α-T is expressed as μg/g wet weight of tissue.

	Fat	Liver	Heart	Muscle	Lung
Vit. E supplemented	190	23	19	11	--
Vit. E deficient	20	5.7	6.2	2.9	4.5

Vitamin E deficiency was invariably accompanied by abnormal RBC hemolysis as measured *in vitro* after addition of H_2O_2. *In vivo*, however, the results of ^{51}Cr-RBC survival determinations have been variable with 2 of 6 patients showing a mild to moderately decreased half-life (17 and 22 days as compared with the normal range of 27-35 days).

Muscle metabolism has been assessed by measuring substances released from this tissue into biological fluids. Creatinuria has been found in 5 of 8 patients thus far examined and correction of this abnormality occurs following α-T therapy. Elevations in muscle-derived enzymes (e.g., serum creatine kinase), which are sensitive indicators of myopathy in animals, have been found only occasionally in a survey of over 30 tocopherol-deficient patients.

Studies are continuing but preliminary conclusions are: 1) no major clinical effects of vitamin E deficiency occur in CF; 2) the presence of creatinuria suggests that α-T supplementation may be desirable in selected cases. (Drs. P. Farrell, R. Wood, J. Bieri, P. di Sant'Agnese, NIAMDD; Dr. V. Fischer, Dept. of Anatomy, Univ. of St. Louis, Mo.)

d) Kallikrein activity in cystic fibrosis

The search for the biochemical defect in cystic fibrosis has frequently centered on exocrine glands and their mechanisms of secretion. Intimately related to this function is the kinin-kallikrein system. Kallikrein is an enzyme that functions to split the kinins from their precursors. It is found in serum and in exocrine secretions. Recently it was reported that kallikrein activity was significantly decreased in the plasma of CF patients and their parents (obligate heterozygotes), and it was speculated that this could represent a fundamental defect. This speculation is as follows: saliva from patients with CF and also synthetic polypeptides such as polylysine are capable of blocking sodium transport in the parotid duct of the rat. Trypsin-like activity in saliva of CF patients has been reported to be decreased, suggesting that perhaps the failure to destroy a normal gene product might result in the inhibition of sodium transport, thus leading to the sweat defect in CF. Since kallikrein is also a proteolytic enzyme, and seems to play a significant role in normal exocrine secretion, perhaps a defect in kallikrein could explain the abnormal exocrine function.

We have begun to study kallikrein activity in the plasma and secretion of patients with cystic fibrosis. We have found the kallikrein activity in plasma to be essentially identical in approximately 25 patients and 25 controls. Data for saliva and sweat are not yet available.

From our data it appears that kallikrein *per se* may not be defective in cystic fibrosis, but it remains to be seen if a defect in some other proteolytic activity (specifically arginine esterase) may be involved in the pathogenesis of this disease. (Dr. J. Pisano, NHLI: Dr. R. Wood, NIAMDD)

e) Jejunal disaccharidase activity in pancreatic insufficiency

Lactase deficiency is said to occur more frequently in patients with CF than in the normal population. In this study jejunal biopsies were done on 8 patients with cystic fibrosis (age 13-41 years) and 9 patients with hereditary pancreatitis (HP) (14-44 years) to assess the relation between disaccharidase activities and pancreatic function. Lactase, trehalase and sucrase activities were measured. Twenty-three controls (21-35 years) were also biopsied.

Pancreatic enzymes were absent and stool fat excretion was greater than 5% in all patients with CF and in 5 of 9 patients with HP. Jejunal lactase activity was significantly lower in the CF group than in the normal group ($p<0.01$). As a group the HP patients did not differ from controls. However, while the 4 HP patients who had normal fat excretion and measurable pancreatic enzymes had normal lactase levels, the 5 HP patients with marked pancreatic insufficiency had very low lactase levels. Trehalase activity was also decreased ($p<0.02$) in the CF group. Trehalase activity in the HP patients paralleled pancreatic function in a manner similar to the lactase activity. Sucrase activity was normal in all patients. These observations suggest that lactase deficiency in patients with steatorrhea is common. The deficiency may not be a primary genetic defect but rather related to the pancreatic insufficiency in those patients. (Dr. Z. Falchuk, C. Sessoms, Dr. L. Taussig, NIAMDD)

II. Clinical Studies

a) Pseudomonas vaccine in cystic fibrosis

One of the major clinical problems in cystic fibrosis is chronic obstructive pulmonary disease, with almost universal colonization of the lung with Pseudomonas aeruginosa. As Pseudomonas is resistant to most antibiotics, other methods of control would be of potential benefit. Recently a purified immunogenic lipopolysaccharide antigen preparation has become available, and has been used with considerable success in patients with unusual susceptibility to Pseudomonas infections, such as massive burns.

We have been investigating the use of this vaccine in patients with CF to determine the optimal doses of vaccine, the duration and extent of the immune response, and the incidence of side effects. Thus far we have found essentially no untoward reactions to the vaccine among our patients other than slight fever and malaise lasting for several hours after the injections. Immune responses are good, with titers rising by one to two orders of magnitude over baseline levels. To date too few patients have been immunized to give any indication of the clinical efficacy of the immunization procedure. This will require a very large cooperative study with several hundred patients. Our preliminary data suggest that such a study would probably be feasible with the immunization procedure as it now exists. (Dr. H. Reynolds, NIAID; Dr. R. Wood, NIAMDD)

b) The use of intrapleural atabrine to prevent recurrent pneumothorax in CF

Pneumothorax is an increasingly frequent complication of the chronic pulmonary disease associated with CF. Episodes are often recurrent and each attack is accompanied by the risk of respiratory insufficiency, pneumonitis, bronchopleural fistula, and even sudden death. Therefore, it has been suggested that prophylactic measures taken after an initial attack and aimed at obliteration of the potential pleural space are indicated to decrease the incidence of recurrence and attending complications.

Quinacrine hydrochloride (Atabrine) has been used successfully in the treatment of neoplastic effusions. When introduced into the pleural space this agent causes local serositis with resulting fibrosis and adhesion of the visceral and parietal pleural surfaces. This mode of therapy may provide a valuable alternative to surgery for the cystic fibrosis patient with severe lung disease and marginal pulmonary reserve.

Intrapleural instillation of quinacrine on two to four successive days until there is local and systemic evidence of inflammation has been used by us with apparent success in preventing recurrences of the pneumothorax in several patients with CF. In one 29-year-old man for 23 months and 10 months following left and right intrapleural quinacrine, respectively, there was no recurrence of pneumothorax on either side. Death was due to pulmonary failure and at autopsy there were dense adhesions completely obliterating the pleural spaces. (Drs.R. Wood, P. Farrell, P. di Sant'Agnese, NIAMDD; Dr. C. McIntosh, NHLI)

c) Genetic pancreatic disorders other than CF

In the biochemical and physiologic studies some of the other genetic pancreatic disorders (e.g., hereditary pancreatitis, pancreatic deficiency with bone marrow dysfunction, etc.) are also being studied in addition to CF. This is especially true of the vitamin E studies and the ones on jejunal disaccharidase activity.

A first phase of clinical investigations on hereditary pancreatitis is now completed and a report recently published in the pediatric literature. Other investigations, both clinical and laboratory ones, are being planned on this disease especially concerning the type of diabetes seen in a number of subjects in the families with hereditary pancreatitis but not having pancreatic involvement. This may give a clue as to the unknown basic genetic defect. (Drs. R. Wood, P. Farrell, P. di Sant'Agnese, NIAMDD)

B. INVESTIGATION IN THE RESPIRATORY DISTRESS SYNDROME (RDS)

The study of the respiratory distress syndrome (RDS) is a new endeavor for our Branch. RDS is quite a common disorder and a leading cause of death among infants in the United States. It is estimated that between 10,000 and 25,000 newborns (especially premature) die of this disorder every year and 30,000 others remain mentally defective because of the lack of oxygen and consequent brain damage caused in the first few hours after birth by RDS.

A great deal has been learned about RDS in the last few years. It has been found that all alveoli in the lung are coated with a lecithin compound (surfactant) which because of its surface active properties opens up the alveoli for breathing at birth and keeps them open thereafter. RDS is apparently caused by an inherited defect in the synthesis of lecithin. In the last couple of years it also has been found that by obtaining samples of amniotic fluid of mothers during gestation and assessing the contents of lecithin which rises rapidly in the last few days of pregnancy, it is possible to predict the patients who are going to be at risk of RDS at birth. This, of course, is especially so if a mother has previously had a child with RDS so that the subsequent ones are apt to get the same manifestations. It was imperative, therefore, to try to find some means of stimulating the production of this lecithin compound in the fetus so that it would be present in adequate amount at birth.

a) Enhancement of lecithin synthesis and phosphorylcholine glyceride transferase activity in the fetal rabbit lung after corticosteroid administration

Kotas and Avery (J. Appl. Physiol., 30:358, 1971) and Motoyama et al. (Pediatrics 48:547, 1971) reported that rabbit fetuses treated with corticosteroid showed evidence of increased pulmonary maturation as determined by pressure-volume curves and surface tension properties. In the present study the effect of steroid on lung lecithin and its synthetic enzymes was measured in premature rabbits. Rabbit fetuses of 23-24 days gestation were injected with 9-fluoro-prednisolone acetate or saline. Three days later the animals were delivered into saline by C-section. In agreement with Kotas and Avery, the steroid treated rabbits showed a slight (15%) decrease in body weight and a moderate (37%) reduction in lung weight. In addition, it was found that the lecithin content of lung parenchyma was elevated in this group from the control value of 70 mg/gm dry weight to 96 mg/gm dry weight ($p<0.005$). Preliminary Studies suggest that the lecithin synthesized is acetone precipitable, and therefore is presumably surface active.

The activities of four enzymes in two lecithin biosynthetic pathways were also examined, namely choline kinase and phosphorylcholine-glyceride transferase (PCGT) of the choline incorporation pathway and methionine adenosyltransferase and phosphatidyl methyl transferase of the methylation route. Of these enzymes, the only significant change in the steroid treated group involved PCGT which was elevated 45%. General protein synthesis was unaffected. These findings suggest that lung PCGT is induced by corticosteroid, leading to increased synthesis of lecithin through the choline incorporation pathway. (Dr. P. Farrell, NIAMDD; Dr. R. Zachman, Univ. of Wisconsin, Madison)

b) Choline phosphotransferase (CPT) and lecithin synthesis in newborn rabbits treated with corticosteroids

Fetal rabbits injected with corticosteroids show elevated lung CPT activity and enhanced lecithin synthesis through the choline incorporation pathway.

To determine if newborns could respond in similar fashion, 28-day gestation rabbits were delivered by C-section and treated with I.M. hydrocortisone (ca. 50 μg/g body weight q 6 hours) or saline. All animals had moderate respiratory distress but maintained a normal blood pH. The steroid treated group differed in no way from the controls in terms of clinical condition and lung appearance. At various intervals from 3-12 hours after birth, the animals were sacrificed and lecithin synthesis measured *in vitro* by incubating lung slices with ^{14}C-choline. Incorporation of isotope into lecithin was the same for 12 control and 12 steroid treated rabbits. Likewise, there was no difference in lung CPT activity.

An improved assay for CPT has been developed in this work and various kinetic features of the enzyme examined. The apparent K_m of rabbit lung CPT for CDP-choline was found to be 3×10^{-5}M. Although a K_m for the diglyceride substrate could not be determined, the enzyme was established as fully saturated in the presence of 1 mM dipalmitin. The absolute CPT activity at saturating substrate concentrations in newborn rabbit lung was 127 picomoles lecithin/min/mg protein. This relatively slow velocity is consistent with a possible role of CPT as the rate limiting enzyme in the choline incorporation pathway. (Dr. P. Farrell, NIAMDD)

c) Cortisol and lung choline phosphotransferase (CPT) activity in the fetal rat after *in utero* decapitation

Reduced lecithin synthesis is present in the lungs of hypophysectomized fetal rats (decapitated at 16 days gestation). Accordingly, the latter animals have been employed to further study the role of cortisol in regulating lung surfactant synthesis.

Measurements of plasma cortisol revealed that fetal rats show a rise beginning on day 19 and reaching a peak of 7.5 μg/100 ml on day 20. Lecithin synthesis as measured by ^{14}C-choline incorporation with lung slices rises in parallel fashion beginning within 24 hours after the change in cortisol. In decapitated fetuses, however, cortisol was found to remain at trace levels throughout gestation. Lung CPT activity was significantly reduced in decapitated fetuses at 22 days gestation from 7.7 ± 0.4 (control mean) to 5.1 ± 0.4 picomoles lecithin/min/mg lung ($p<0.001$). Early attempts to raise the enzyme level by injecting the decapitated animals with dexamethasone have been partially successful, a group of 5 showing a mean CPT of 7.4. Nuclear dexamethasone receptors were found to be quite adequate in the decapitated fetus, peaking on day 19 along with the controls.

These findings support the hypothesis that in the fetus cortisol plays a key role in controlling lung CPT and regulating lecithin synthesis and suggest that hydrocortisone may be beneficial as a specific therapy in the respiratory distress syndrome. (Dr. P. Farrell, NIAMDD; Dr. W. Blackburn, Penn. State Univ. Col. of Med., Hershey, Pennsylvania)

Serial No. NIAMDD-PMB-1c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Metabolic, Physiological, and Biochemical Studies in Cystic Fibrosis.

Previous Serial No.: None

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: Philip M. Farrell, M.D., Ph.D., Robert E. Wood, M.D., Ph.D., J. Charles Pallavicini, Ph.D., Michael Klagsbrun, Ph.D., John G. Bieri, Ph.D., NIAMDD; John J. Pisano, M.D., NHLI.

Cooperating Units: Laboratory of Biochemical Pharmacology, NIAMDD; Laboratory of Nutrition and Endocrinology, NIAMDD; Vernon Fischer, M.D., St. Louis University Medical School; Experimental Therapeutics Branch, NHLI.

Man Years: Total: 3.5
Professional: 1.5
Other: 2.0

Project Description:

Objectives:

1. Studies in tissue culture fibroblasts: Growth rate. Although the primary biochemical error in cystic fibrosis (CF) remains unknown, several lines of evidence suggest that fibroblasts from CF patients are metabolically abnormal. In order to evaluate these cells comprehensively a fundamental approach is being applied to examine basic biological and biochemical parameters. Specifically, growth rate of the fibroblast and protein synthesis at various stages of growth is being determined.

2. Studies in tissue culture fibroblasts: RNA methylation. It has been reported that fibroblasts in tissue culture from patients with CF show decreased methylation of total RNA. In addition, it has been claimed that fibroblasts from obligate heterozygotes with CF also manifest reduced RNA methylation to values intermediate between controls and CF homozygotes. Such an alteration might have important metabolic implications. Therefore, it was proposed to reinvestigate RNA methylation in CF fibroblasts by applying improved techniques of cell culture, RNA labeling, and RNA isolation and fractionation.

3. Effects of vitamin E deficiency in man: The biological manifestations of vitamin E deficiency are more protean in various animal species than for any other nutrient. However, man has not been shown conclusively to develop clinical symptoms of vitamin E deficiency. Patients with CF represent the ideal experimental model of profound vitamin E deficiency, often present for many years. Study with sophisticated methods in a comprehensive approach should permit a better definition of the changes in metabolism which are likely produced by a deficiency in this vitamin. It is, therefore, proposed to evaluate several biochemical and morphological parameters of vitamin E deficiency in a group of 85 patients with CF and other pancreatic insufficiency states.

4. Kallikrein activity in cystic fibrosis: Kallikrein activity is reportedly decreased in the plasma of patients with CF. Because the deficiency of a proteolytic enzyme might explain at least part of the pathogenesis of CF, we sought confirmation of this observation.

Methods Employed:

1. Rate of growth in normal versus CF fibroblasts is being measured by both cell counts and DNA assays. Protein synthesis is being assessed by measuring protein concentration on a cellular basis by the standard Lowry method. In addition, more direct measurements of protein synthesis are being performed by incubating cultured fibroblasts with ^{14}C-leucine during various stages of growth. Isolation of protein as TCA precipitable material and leucine by ion exchange chromatography permits determination of specific isotopic activity and calculation of protein synthetic rates.

2. Control and CF fibroblasts were cultured in the presence of L-(methyl-^{14}C) methionine and 5-^{3}H uridine. RNA was then extracted and fractionated by sodium dodecyl sulfate sucrose density gradient centrifugation. Ratios of $^{14}C/^{3}H$ incorporation serve as a measure of the extent of RNA methylation. In addition, methylated base composition of human fibroblast tRNA was identified by two-dimensional chromatography. Methylation rates were also measured *in vitro* by incubating fibroblast extracts with E. *coli* tRNA and either S-adenosyl (methyl-^{3}H) methionine or S-adenosyl (methyl-^{14}C) methionine.

3. Vitamin E as the abundant α-tocopherol isomer has been measured spectrophotometrically after isolation by thin layer chromatography. Erythrocyte hemolysis has been measured after addition of hydrogen peroxide to washed RBC suspensions. Creatine excretion has been determined utilizing a specific enzymatic technique for the metabolite. Skeletal and myocardial muscle has been examined histologically and histochemically. Various muscle-derived plasma enzymes, e.g., creatine kinase, have been determined by standard techniques.

4. Measurements of kallikrein levels were performed using a new and highly specific assay. Plasma was collected from CF patients and age-matched controls. Urine, sweat, and saliva are now being collected for study by several different assay methods.

Major Findings:

1. Results show comparable rates of cell proliferation in both control and CF cell lines. Additionally, the DNA content per cell was similar. The protein content, however, as expressed on a DNA basis, was consistently lower in CF cell lines during the logarithmic phase of growth ($p<0.01$). In the post confluent phase, protein concentration reached levels similar to the control cells. Direct measurements of protein synthesis are in progress.

2. Examination of $^{14}C/^{3}H$ incorporation revealed that tRNA, 18S rRNA, and 28S rRNA of CF cells were methylated to a normal extent. Methylated base composition was found to be the same in tRNA and rRNA for CF and control cells. Furthermore, this pattern was identical to that previously found for RNA from yeast, various animal tissues, and HeLa cells. Measurement of methylation *in vitro* revealed statistically unaltered rates for CF fibroblast extracts. Identification of the bases again revealed a normal pattern for CF cells.

3. Cystic fibrosis patients with pancreatic achylia have uniformly shown decreased plasma α-tocopherol. Abnormal erythrocyte hemolysis *in vitro* has been found in all individuals with less than 400 μg/100 ml α-tocopherol. Excessive hemolysis correlates in a sigmoid fashion with plasma α-tocopherol. The majority of patients examined thus far have shown excessive excretion of creatine. However, the activities of muscle-derived enzymes have only been occasionally elevated.

4. There was no significant difference in kallikrein levels in plasma or urine of controls versus CF patients. Data are not yet available for sweat and saliva.

Significance to Biomedical Research:

1. Determination of cell growth kinetics and the rate of cellular protein synthesis is not feasible in patients with CF. Tissue cultured fibroblasts, on the other hand, offer a useful system and presumably manifest the metabolic error of CF. The finding of normal growth rate is useful to the design of other experiments and by extrapolation to the patient suggests that cells in CF patients are fundamentally capable of normal growth. The apparently reduced rate of protein synthesis in early growth, if substantiated by direct isotopic measurements of protein turnover (in progress), might have important implications for the affected patients and may provide a clue as to the primary metabolic error.

2. These results clearly indicate that all tRNA methylase enzymes are fully active in CF fibroblasts. The extent of tRNA and rRNA methylation is unaltered in CF and there are no differences in methylated base composition. Application of these improved techniques over those used by other investigators has shown that decreased RNA methylation is not a fundamental feature of CF fibroblasts, if present at all. Measurement of RNA methylation in CF fibroblasts and lymphocytes should not be employed as a means of carrier detection.

3. Comprehensive investigation of vitamin E deficiency in cystic fibrosis may enable us to answer the unresolved issue of whether or not vitamin E deficiency in man causes metabolic derangements. Results thus far suggest that α-tocopherol deficiency is accompanied by definite abnormalities.

4. Exocrine glands are a major site of involvement in CF; kallikrein is intimately related to exocrine function. It appears from this study that a defect in kallikrein function cannot explain the pathogenesis of CF. In the published reports of decreased kallikrein activity much less specific methods were employed and it is possible that some similar enzyme, but not kallikrein, may be involved.

Honors and Awards: None

Publications:

Klagsbrun, M., and Farrell, P.M.: The methylation of RNA in fibroblasts of normal volunteers and patients with cystic fibrosis. In Mangos, J.A., and Talamo, R.C. (eds.): Fundamental Problems of Cystic Fibrosis and Related Diseases. Miami, Florida, Symposia Specialists, 1973 (in press).

Serial No. NIAMDD-PMB-2c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Clinical Studies in Cystic Fibrosis

Previous Serial No.: None

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: Robert E. Wood, M.D., Ph.D., Philip M. Farrell, M.D., Ph.D., NIAMDD; Herbert Y. Reynolds, M.D., NIAID; Charles L. McIntosh, M.D., NHLI.

Cooperating Units: Laboratory of Clinical Investigation, NIAID (480); Surgical Branch, NHLI.

Man Years: Total: 2.25
Professional: 1.5
Other: 0.75

Project Description:

Objectives:

1. A study of Pseudomonas vaccine in cystic fibrosis (CF): To measure antibody responses to pulmonary colonization and infection with Pseudomonas species and to immunization with a vaccine prepared against Pseudomonas. To demonstrate safety and possible clinical efficacy of Pseudomonas vaccine.

2. Intrapleural quinacrine hydrochloride as a therapeutic agent for recurrent pneumothorax in cystic fibrosis: To demonstrate a simple and safe method to prevent the recurrence of pneumothorax in cystic fibrosis by promoting the formation of pleural adhesions by nonsurgical means.

Methods Employed:

1. Patients with CF are given a series of injections of heptavalent Pseudomonas vaccine with monitoring of clinical and immunological responses. Antibody titers are measured in serum and bronchial secretions. Pulmonary colonization is being followed by repeated sputum cultures with serotyping of Pseudomonas strains.

2. Patients hospitalized with recurrent pneumothorax are given intrapleural instillations of quinacrine hydrochloride through a chest tube. The instillations are repeated daily until there is adequate evidence of an inflammatory reaction in the pleural space.

Major Findings:

1. Pseudomonas vaccine is well tolerated by patients with cystic fibrosis with only slight local or systemic reactions. Antibody levels rise by one to two orders of magnitude following injection. We are currently collecting data on the persistence of the antibody titers and the need for and efficacy of booster immunizations. The sample size at present is too small to meaningfully evaluate clinical response.

2. This form of treatment seems successful in producing significant pleural adhesions as judged by autopsy findings in one case and by the lack of recurrence of pneumothorax after therapy in other cases. Some adverse effects have been noted but the method seems much more benign than surgical obliteration of the pleural space.

Significance to Biomedical Research:

1. Most patients with CF have chronic pulmonary colonization with Pseudomonas, an organism which is notoriously resistant to most antibiotics. The development of an effective immunization program against Pseudomonas species could be of significant therapeutic benefit to patients with cystic fibrosis.

2. Recurrent pneumothorax is a frequent and life threatening complication of CF in older patients. The standard method of treatment for recurrent pneumothorax is obliteration of the pleural space usually by surgical means. Since pulmonary surgery on patients with CF is quite risky, an effective nonsurgical means of pleural obliteration would be of considerable therapeutic importance.

Honors and Awards: None

Publications: Kattwinkel, J., Taussig, L., McIntosh, C., di Sant'Agnese, P., Boat, T., and Wood, R.: Intrapleural quinacrine in recurrent pneumothorax in cystic fibrosis. JAMA, 1973 (in press).

Serial No. NIAMDD-PMB-3c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Pulmonary Lecithin Synthesis as Related to the Respiratory Distress Syndrome.

Previous Serial No.: None

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: Philip M. Farrell, M.D., Ph.D., NIAMDD; Ronald A. Chez, M.D., NICHD.

Cooperating Units: Pregnancy Research Branch, NICHD; W. Blackburn, M.D., Hershey Medical Center, Hershey, Pa.; George W. Kittinger, Ph.D., Oregon Primate Center, Beaverton, Oregon.

Man Years: Total: 3.25
Professional: 1.0
Other: 2.25

Project Description:

Objectives:

Respiratory distress syndrome (RDS) occurs primarily in premature newborns and is characterized by progressive pulmonary atelectasis. The disorder has a higher incidence than any other pediatric pulmonary disease of metabolic origin. It has become quite clear in the last several years that a decrease in the concentration of lung surfactant (composed primarily of disaturated lecithin) is causally related to RDS. By implication, a decreased rate of pulmonary lecithin synthesis is the likely pathogenetic factor in the disease. Although the etiology of the disorder is not known, a metabolic error (inborn error of metabolism) in lecithin synthesis must be highly suspect. A rational therapeutic approach, therefore, must be aimed at increasing the rate of surfactant synthesis. The objectives of this project are as follows: The principal *de novo* pathway for lung lecithin synthesis will be determined by measuring the rate of lecithin formation from either choline incorporation or phosphatidylethanolamine methylation. Since some investigators have claimed that pronounced species specificity occurs in regard to the relative contributions of the two pathways, several species (both adult and fetus) are being examined, including human, monkey, rabbit, and rat. The key question of what factors regulate lung lecithin synthesis is being approached by studies on the enzymes of the two pathways and by determining levels of substrate (metabolites) in the pathways. By determining the changes in rate of lecithin synthesis as a function of gestation, the importance of regulatory factors will be determined. The role of corticosteroids in controlling lung maturation and lung lecithin synthesis is being assessed in both fetal and newborn animals.

Methods Employed:

Lecithin synthesis is measured by thin layer chromatography and by isotopic incorporation studies with lecithin precursors. Activities of the enzymes in the choline incorporation pathway (choline kinase, cytidyl transferase, and choline phosphotransferase) are measured using isotopically labeled substrates. Rabbit and rat fetuses have been injected with corticosteroids *in utero* and followed for various periods of time with respect to lecithin synthesis and enzyme activities. Additionally, decapitated rat fetuses, corticosterone deficient and low in lung lecithin, have been utilized as a model of hormone deficiency. Inhibitors of protein synthesis, such as cycloheximide and actinomycin D have been employed in order to define the specific site of action for corticosteroids in fetal lung lecithin synthesis.

Major Findings:

Lung lecithin concentration and isotopically-measured rate of lecithin synthesis increases during the latter 10-20% of gestation for both fetal rat and rabbit. The principal pathway for lung lecithin synthesis appears to be the choline incorporation route rather than phosphatidylethanolamine methylation. Administration of corticosteroids to the fetus enhances the rate of lung synthesis within six hours after injection and increases the activity of choline phosphotransferase (CPT). These changes may be blocked by prior administration of cycloheximide but not actinomycin D. The latter data suggest that corticosteroids act to promote mRNA translation for CPT rather than DNA transcription. Corticosterone deficient decapitated rat fetuses show low lung choline phosphotransferase activity which may be increased to normal within six hours after a physiological dose of dexamethasone.

Various kinetic properties of the three enzymes in the choline incorporation pathway have been determined. By full order of magnitude, choline phosphotransferase shows the lowest specific activity of the three enzymes, and thus appears to be the rate limiting catalyst in the pathway.

Significance to Biomedical Research:

Prior to 1971, despite the overwhelming importance of respiratory distress syndrome as a community health problem, very little was known in general about lung biochemistry and in particular about the synthesis of surface active lecithin by fetal and newborn lungs. Application of fundamental biochemical techniques to this problem is the most reliable approach and thus far has been quite fruitful. In order to identify the metabolic error responsible for RDS, basic features of the lung lecithin pathway must be determined, including those factors which play a role in regulation. These studies have thus far indicated that corticosteroids are powerful inducers of lung lecithin synthesis, an action mediated by enzyme induction. The induced enzyme, choline phosphotransferase, is the rate limiting catalyst of the pathway and may play a role in the etiology of RDS.

Honors and Awards: None

Publications:

Farrell, P., and Zachman, R.: Corticosteroids as primary therapeutic agents in respiratory distress syndrome. Pediatrics, 1973 (in press).

CLINICAL HEMATOLOGY BRANCH

I. Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

1. The Metabolic Response of Platelet to Thrombin and Other Platelet Aggregating Agents

When platelets are acted on by thrombin or other physiologic agents that cause platelet aggregation (which is closely related to formation of a hemostatic platelet plug *in vivo*) the platelets release cytoplasmic granules that contain adenine nucleotides, calcium, and vasoactive amines. This "release" reaction is energy dependent and is accompanied by a marked metabolic response in platelets. Our initial findings that platelets contain a highly active adenyl cyclase-cyclic AMP system that responds to physiologic agents has led to the concept that changes in cyclic AMP act as a second messenger in platelets as it apparently does in response to hormones in other secretory cells. In most cells studied, increases in cyclic AMP have been associated with stimulation of cell function but in platelets the opposite appears to be true. Although some workers have reported that *increases* in cyclic AMP are associated with the release reaction and aggregation, it is clear from our work this year that agents that *increase* cyclic AMP inhibit metabolic responses of platelets and *prevent* the release reaction and aggregation whereas agents that *decrease* cyclic AMP *initiate* release and aggregation. Prostaglandin E_1 which increases adenyl cyclase activity and theophylline which inhibits phosphodiesterase markedly increase cyclic AMP in platelets and prevent both release and aggregation. The effects of PGE_1 and theophylline are additive. The marked increases in lactate production and CO_2 formation stimulated in platelets by aggregating agents are inhibited by PGE_1 and theophylline. Thrombin and other aggregating agents which cause the platelet release reaction produce an initial decrease in platelet adenyl cyclase activity and a corresponding decrease in cyclic AMP. This occurs both in platelet suspensions and in the basal metabolic state or in suspensions stimulated by PGE_1. After the initial decrease in cyclic AMP and adenyl cyclase there is a rapid return to control levels and in some instances an apparent compensatory over-production of cyclic AMP. Previous studies reported by other investigators were in error because measurements were made long after the compensatory period, whereas our findings are more pertinent because observations were made in the 15 second to 30 second interval during which the release reaction takes place and aggregation is initiated. The other metabolic responses such as increased CO_2 and lactate formation also begin within the first half minute of observation.

2. Platelet Phosphodiesterase Activity

Since it is now known that platelet function is intimately associated with changes in the level of intracellular cyclic AMP, it was of interest to see whether one aspect of the control of function involved phosphodiesterase activity. There are reports suggesting that imidazole promotes platelet aggregation and platelet release by stimulating phosphodiesterase. Although phosphodiesterase of other organs appears to increase in the presence of imidazole, we have found that platelet phosphodiesterase does not respond in this way. There are a number of studies of plasma cyclic AMP either in association with various diseases of various physiologic states such as shock or exercise. We have found that platelet phosphodiesterase is present in anticoagulated or clotted blood in amounts that would rapidly clear cyclic AMP from serum or plasma. It is apparent that the level of platelets, the anticoagulant used, and the treatment of blood to preserve platelet integrity as well as the period of time permitted for blood to clot are all important factors in determining plasma cyclic AMP levels.

3. Thrombasthenia

Thrombasthenia is a rare inherited qualitative platelet defect characterized by an infinite bleeding time, normal platelet numbers, lack of clot retraction, and in some instances, absence of platelet aggregation in response to a variety of physiologic stimuli. The cause of the defective platelet function is not understood. We've studied two cases of thrombasthenia, assessing platelet metabolism and function and response of the patients to transfusion of normal platelets in each case. In each case, clot retraction and platelet aggregation were defective, although platelet metabolism as judged by lactate production, glucose oxidation, nucleotide release, and lipid synthesis were all normal. There was no plasma inhibitor of platelet function and the thrombasthenic platelets did not react normally in normal plasma. Normal platelets infused in thrombasthenic patients provided only slight transient correction of bleeding time and clot retraction, suggesting that the patients' unaggregated platelets interfere with the function of normal platelets. Since treatment of life-threatening bleeding in the past has been unsuccessful, even with massive platelet transfusion, this finding suggests that the patient's own platelets should be removed by plasmapheresis before administering large amounts of normal platelets therapeutically.

4. Platelet and Megakaryocyte Acid Phosphatase

Although it is known that platelets contain acid phosphatase, little is known concerning the biochemical characteristics of this enzyme in platelets and there is essentially no information on megakaryocytic acid phosphatase. In histochemical and biochemical studies of platelet enzymes aimed at attempting to identify platelet age and function by possible variations in their enzymatic constituents, acid phosphatase was isolated from platelets and its stability determined under various physicochemical conditions as well as its electrophoretic characteristics in various media. The enzymatic activity was found for the first time to be a function of three isoenzymes distinguishable electrophoretically and by differential inactivation by organic solvents such as acetone and formaldehyde. Applying the same techniques to megakaryocytes but using a tissue staining technique rather than an enzymatic assay, it was found that megakaryocytes contained the same three isoenzymes but in different proportion than platelets. By histochemical stains, individual platelets had different amounts of the three isoenzymes. Current studies are underway to determine whether the isoenzyme stable in inorganic solvents is found in higher concentration in younger compared to older platelets. It may be possible to correlate specific patterns of the three enzymes in platelets and megakaryocytes with platelet age and turnover to provide a new approach for differentiating diseases of platelet production and destruction.

II. Study of the Immunology of Blood Cell Deficiencies

1. Metabolic Effects of Antibodies on Platelets

For many years it has been known that platelets discharge cellular materials and undergo clumping in response to immune stimuli such as antigen-antibody complexes and antiplatelet antibodies. The loss of cellular material in immune responses generally has been considered a consequence of cell lysis by complement, and the clumping has been considered an agglutination phenomenon mediated by antibody bridging between cells. We have found that immune agents stimulate platelets to respond in the same way that they respond to physiologic stimuli such as thrombin, collagen, and epinephrine, by a metabolically dependent release reaction followed by aggregation. That is, the metabolic and functional responses induced in platelets by antiplatelet antibodies are similar in all respects to responses produced by other physiologic substances rather than unique complement or antibody mediated phenomena. The release of adenine nucleotides from platelets by antigen-antibody complexes or antiplatelet antibodies occur at the same rate and to the same degree as the release initiated by thrombin or other physiologic stimuli and are proportional to antibody concentration. Antibody responses are dependent on ionic calcium and an as yet unidentified plasma factor that are also required with other physiologic stimuli. The plasma (or serum) factor is not complement for the reaction in heated plasma and with antibody eluates that do not contain complement components. Antibodies stimulate CO_2 formation and lactate production in platelets to the same degree and in the same time sequence as thrombin. Similarly the response of platelet adenyl cyclase and cyclic AMP is identical to that following stimulation by thrombin (see Project 1c). Metabolic inhibitors or stimulators such as prostaglandin E_1 or theophylline influence the response to antibodies in the same way that they affect response to other physiologic agents. Platelets affected by antibodies are sequestered by the reticuloendothelial system rather than lysed in the circulation. How the RE system recognizes cells altered by the attachment of relatively few gammaglobulin molecules is not known. The membrane and metabolic changes produced by antibodies rather than attachment of antibodies per se could be the basis for RE recognition and subsequent destruction of these cells. Therefore, inhibitors of these antibody-induced changes may prevent the sequestration of immunologically altered platelets. Drugs that inhibit the release reaction and aggregation such as dipyridamole and sulfinpyrazone or agents that increase cyclic AMP such as theophylline or PGE_1 deserve consideration as potential therapeutic agents in immunologic platelet disorders. The demonstration that platelet membranes and metabolism are affected by anticellular antibodies that do not destroy platelets bur rather cause subtle membrane changes may help explain the processes involved in immunologic surveilance within the body, particularly for control of cell mutation.

2. The Platelet Release Reaction as a Measure of Antiplatelet Antibodies

Because many antiplatelet isoantibodies and the antiplatelet factor responsible for ITP cannot be measured by conventional immunologic techniques, we have attempted to utilize the platelet release reaction as a sensitive indicator of immune reactions involving platelets. For reasons that are not yet clear, antiplatelet antibodies that do not obviously disrupt cellular organization of the platelet as evidenced by morphologic criteria or change most platelet function tests nevertheless result in loss of material contained in platelet granules. Two of the granular substances most easily and sensitively measured are adenine nucleotides and serotonin. We have found that antibodies with complement fixing properties are most active in causing release of platelet granular material and initiating platelet aggregation. This occurs even when the antibodies are used at low concentrations that do not support detectable complement fixation and even when these antibodies act in the absence of complement e.g., after heat inactivation of serum. Antibodies that do not have complement fixing properties can also be detected by the release reaction but these antibodies do not usually cause subsequent platelet aggregation. ITP factor (which we have previously identified as a gammaglobulin in most instances) did not have the same effect as isoantibodies in the release system. Only three of thirty patients with ITP had in their plasma a factor that caused platelet release of serotonin or adenine nucleotides. These findings are in contrast to those of others who suggest that the ITP factor has *in vitro* reactions similar to those of isoantibodies. The studies of others may be in error because of thrombin-like activity in poorly clotted thrombocytopenic blood or because of releasing factors that developed in citrated plasma stored for many years at -20°.

3. A New, Highly Sensitive Test for the Antigen of Viral Hepatitis

The specific antigen popularly known as Australia antigen is in the serum of many patients with viral hepatitis and also in healthy carriers of the virus who transmit the disease in blood they donate. There is need of a simple, rapid and sensitive test to eliminate blood donors who may transmit the disease. At the present time, counter electrophoresis and radioimmunoassay are the most frequently used tests for this antigen. The most sensitive test is radioimmunoassay but it is plagued by many false-positive reactions and requires expensive equipment and a time delay between collection and use of blood that is unacceptable in many blood banks. By using a hemagglutination process similar to the one develop by us for detecting antibody against Australia antigen, we have developed a simple and sensitive technique that matches the radioimmunoassay in sensitivity for detecting the hepatitis antigen. The technique is additionally valuable because it requires no special apparatus, is performed in a short period of time, and is an extremely stable reagent that can be shipped without difficulty. Moreover, the technique does not give false-positives as does the radioimmunoassay. This technique will probably replace the radioimmunoassay as a more sensitive and accurate method of screening prospective blood donors.

4. Application of Hemagglutination Techniques to Various Problems of a Practical Clinical Nature

One of the important problems in public health currently is drug addiction. The tests for various drugs, notably for morphine and its derivatives and for barbiturates and related compounds are tedious in that they usually require concentrations of samples presented and elaborate equipment for measuring electron spin resonance or performing thin layer chromatography. By coupling drug to carrier protein and making antibodies against the drug in animals, it was possible to develop a hemagglutination inhibition for drug. In the test the protein-drug complex is attached to a preserved red cell as the inert particle and the basis of the test is inhibition of antibody against the drug by free drug which may be present in the blood, secretions or excreta of the individual being tested. In this way we have found it possible to measure amounts of morphine or its derivatives in urine several days after the usual therapeutic or narcotic dose. This type of measurement is most important for public health purposes in screening individuals suspected of being drug users. We feel that this technique is more valuable than any of the others currently available because of its simplicity and specificity.

Serial No. NIAMDD-CHB-1c
1. Clinical Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

Previous Serial Number: SAME

Principal Investigator: Dr. N. Raphael Shulman

Other Investigators: Dr. Roger Lange
Dr. Peter Tomasulo

Man Years
Total: 3.0
Professional: 1.5
Other: 1.5

Project Description:

Objectives:

Study of the reactions and interactions of coagulation factors in vitro and in vivo to define further the nature of the blood coagulation mechanism, to determine factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis, and to develop better forms of therapy for these diseases.

Methods Employed:

Methods of protein purification and characterization, techniques of enzymology applied primarily to proteolytic enzymes and their inhibitors, kinetic analyses of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactively labeled substrates.

Honors and Awards: none

Publications:

1. Gockerman, J.P., and Shulman, N.R.: Isoantibody specificity in post-transfusion purpura. Blood 41:6, 1973.

2. Lange, R.F., Shulman, N.R., Tomasulo, P.A., and Coleman, C.N.: Metabolic effects of antibodies on platelets. Trans. Assoc. Amer. Phys., 1973 (in press).

3. Counts, R.B., Silvergleid, A.J., and Shulman, N.R.: Thrombasthenia: studies of platelet physiology and of response to platelet transfusion. Nature, 1973 (in press).

4. Marchesi, S.L., Shulman, N.R., and Gralnick, H.R.: Studies on the purification and characterization of human factor VIII. J. Clin. Invest. 51:2151, 1973.

5. Counts, R.B., and Shulman, N.R.: Thrombin and collagen-induced aggregation of human platelets. Aggregation is not mediated by released ADP. Blood, 1973 (in press).

Serial No. NIAMDD-CHB-2c
1. Clinical Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Study of the Immunology of Blood Cell Deficiencies

Previous Serial Number: SAME

Principal Investigator: Dr. N. Raphael Shulman

Other Investigators: Dr. Roger Lange
Dr. Peter Tomasulo

Man Years
Total: 3.0
Professional: 1.5
Other: 1.5

Project Description:

Objectives:

To study the nature of immunologic reactions which result in formation of antibodies against autologous blood cells, and to determine the significance of immunity in development of "idiopathic" blood cell deficiency states. Of special interest are the biochemical reactions which result in formation of complexes between cells, antibodies, and drug haptenes and the physiologic processes which result in sequestration of cells with attached antibodies. In recent years our work on identifying inherited leukocyte and platelet isoantigens has led to a study of the significance of these antigens in hetero- and homo-transplantation and in possibilities of developing immunologic therapy against malignancies. Immunologic techniques have been used to develop diagnostic techniques for evaluating the infectious diseases, hepatitis B and toxoplasmosis.

Methods Employed:

Techniques of quantitative immunochemistry, including preparation and physicochemical characterization of purified antibodies and antigens, micro-analyses for nitrogen, histamine, and alkaloid drugs, quantitative measurements of complement fixation, cellular agglutination and precipitin reactions, immuno-electrophoresis, methods of provoking antibody responses in man and animals, and isotopic and fluorescent labeling applied to antigens and antibodies. Tissue culture techniques, red cell agglutination techniques and lymphocyte transformation tests, including radioautography. Methods of hemagglutination involving attachment of various antigens to erythrocytes by chemical coupling.

Honors and Awards: none

Publications:

1. Shulman, N.R.: Complement fixation tests. In Vyas, G.N., Perkins, H.A., and Schmid, R.S. (eds.): Hepatitis and Blood Transfusion. New York, Grune & Stratton, 1972, pp. 133-135.

2. Hirschman, R.J., and Shulman, N.R.: Utilization of the platelet release reaction to measure ITP factor and platelet antibodies. Trans. Assoc. Amer. Phys. 85:325, 1972.

3. Shulman, N.R., Lange, R.F., Knepp, C.S., and Coleman, C.N.: Occurrence of HBAg and antigens in excreta and secretions. In Vyas, G.N., Perkins, H.A., and Schmid, R.S. (eds.): Hepatitis and Blood Transfusion. New York, Grune & Stratton, 1972, pp. 257-268.

4. Barker, L.F., Peterson, M.R., Shulman, N.R., and Murray, R.: Antibody responses in viral hepatitis, type B. J. Amer. Med. Assoc. 223:10, 1973.

5. Shulman, N.R.: Complement fixation and hemagglutination techniques for measuring hepatitis-associated antigen and antibody. In Prier, J.E., and Friedman, H. (eds.): Australia Antigen. Baltimore, Md., University Park Press, 1973, pp. 83-94.

6. Shulman, N.R.: Drug induced antibodies to blood cells. In Dimitrov and Nodine (eds.): Drugs and Hematologic Reactions. New York, Grune & Stratton, 1973 (in press).

EXTRAMURAL PROGRAMS

DEPUTY DIRECTOR'S STATEMENT

Reorganization of the Extramural Programs

The reorganization of the NIAMDD extramural programs into "clusters," as discussed in last year's annual report, was approved both by the Office of the Director, NIH, and the Office of the Assistant Secretary for Health. The full implementation of the reorganization plan was not feasible, however, during FY 1973 because of the reduction in personnel slots available to the Institute. The Digestive Diseases and Nutrition cluster was organized under the leadership of Dr. Lionel M. Bernstein as Associate Director for Digestive Diseases and Nutrition. The position of Associate Director for Extramural Program Activities was assumed by Dr. Edward P. Offutt, with the responsibility for generally coordinating the administrative and operating functions of the extramural programs. The programs other than DD and N were organized very "loosely" into their clusters, but with no firm administrative arrangement by which they could adequately function in this capacity.

Thus, by the end of FY 1973, the organization of the extramural programs consisted of a Digestive Diseases and Nutrition cluster, under an Associate Director; an Arthritis, Orthopedics, and Dermatology cluster; a Diabetes, Endocrinology, and Metabolism cluster; a Kidney Disease, Urology, and Hematology cluster; and a division under the Associate Director for Extramural Program Activities, which included the activities of grants management, data analysis, application processing, and various administrative functions under the Administrative Officer of the Extramural Programs.

The Institute's Deputy Director assumed particular responsibility for coordinating the activities of the extramural programs.

Training Programs

The most significant change which occurred in the extramural programs, not only in this Institute but overall in the National Institutes of Health, resulted from the decision by the Administration that no new commitments would be made in any of the training mechanisms, including training grants, fellowships, etc., after January 29, 1973. Mechanisms were established by the NIH for phasing out of the commitments in training which had already been established. Institute plans in training will be further discussed in the report of the Training Officer.

Funding of Research Grants

In January 1973 it became known that there would be a significant decrease of funds available for competing grants below that which had been anticipated in the "original" President's FY 1973 budget. Thus the funds available for competing grants during the last half of the fiscal year would fund only approximately ten percent of the approved applications. In view of these circumstances, the Office of the Director, NIH, approved the request of the NIAMDD that an exception be made to NIH policy regarding reduction of committed awards by allowing the NIAMDD to reduce its noncompeting awards made during the last half of FY 1973 by an average of ten percent. This reduction applied not only to the FY 1973 awards but also the "commitments" for the remaining project period. The funds thus recovered enabled the Institute to award approximately 28 percent of the approved applications throughout the year, with preference being given to competing continuations of high scientific merit.

Centers

In conjunction with Council, the Institute sought to establish "center" programs, both in Diabetes and in some aspects of Digestive Diseases and Nutrition. These programs will be discussed in greater detail under the appropriate "cluster" reports. It should be pointed out, however, that the programs were initiated at a time when it was expected that a larger amount of funds would be available for competing grants than that which was finally available for expenditure. Thus, although applications were received and review undertaken for consideration by the June 1973 Council, no funding of any center project was possible during FY 1973. It was anticipated, however, that funding would be possible for a very limited number of centers during the early part of FY 1974.

Of interest was the fact that the types of centers being proposed in each program area were quite different. The Diabetes Centers were conceived as "core" grants to which individual projects, program projects, etc., would be appended through the usual review system. The Digestive Diseases and Nutrition Centers, on the other hand, were conceived as large, multi-project centers designed to stimulate effort in a given area.

Cooley's Anemia

The signing of PL 92-414, the "National Cooley's Anemia Control Act," on August 29, 1973, resulted in the initiation of a formal inter-Institute coordinating activity at NIH in this area. While NIAMDD had been the chief supporter of research in Cooley's anemia, related projects were funded by a number of the other NIH components, particularly the National Heart and Lung Institute which had the responsibility for the intramural activities in this disease. Although no earmarking of funds for this disease was included in the appropriation, the NIAMDD, through the support of workshops and by contract, was stimulating effort in this area. This will be covered more extensively in the report on the Hematology Program.

Evaluation Studies

A number of evaluation studies were initiated by the Institute's extramural staff as a means of obtaining information upon which better program planning could be based. Each of these will be discussed in greater detail under the appropriate section but, briefly, these consisted of the following.

A very large evaluation effort was undertaken in Digestive Diseases, utilizing contract funds approved for the Department's evaluation effort.

An evaluation of the National Pituitary Agency was undertaken by the Endocrinology Program, also utilizing contract funds approved for the Departmental evaluation effort.

An evaluation of the impact of the phase-out of training programs on training in the clinical research areas was undertaken by the Institute, under the immediate leadership of Dr. William H. Batchelor, the Institute's Training Officer, in conjunction with a select number of medical schools.

Evaluation of the research needs in selected areas of arthritis and orthopedics was undertaken in conjunction with the Arthritis Foundation and the American Academy of Orthopaedic Surgeons.

Public Advisory Committees

Fiscal Year 1973 saw a markedly increased activity on the part of the Office of Management and Budget and the DHEW in responding to Administration and Congressional directives through the Federal Advisory Committee Act PL 92-463 with regard to review and evaluation of the public advisory committees. As a result of this review and the Administration's decision with regard to the training programs, the Training Committees of the Institute were recommended for dissolution.

In compliance with provisions of PL 92-305 which was signed on May 19, 1972, there was established a subcommittee for digestive diseases of the National Arthritis, Metabolism, and Digestive Diseases (NAMDD) Advisory Council. To fulfill the intent of this law, permission was asked and given to expand the membership of the Council from 16 to 18 in order that two additional experts in gastroenterology could be appointed. Although to date no action has been taken by the Department on the nominations of these two additional members, the subcommittee, enlarged to include both digestive diseases and nutrition, has been established. The subcommittee held its first meeting in conjunction with the November 1972 meeting of the Council and it is generally agreed that it has been a successful innovation. It is likely that additional subcommittees covering the other clusters will be formed as needed.

It had been proposed and permission had been obtained from the Assistant Secretary for Health to establish a technical advisory committee, known as the NIH Ad Hoc Technical Advisory Committee on Cooley's Anemia, to provide advice to the Director, NIH, on matters relating to this area. This committee would be housed for "care and feeding" within the NIAMDD. The current Departmental review of public advisory committees has held up action, for the time being, on the establishment of this committee.

Equal Employment Opportunity

A determined effort has been made during this year to improve the participation of the extramural program staff in the equal employment opportunity activities in order to reach the goals set by the Institute Director. As part of this effort a representative of Grades 1 through 9 was elected who, amongst other activities, attends the weekly extramural staff meeting as a representative of this group of employees. Similarly, the Personnel Advisory Committee in extramural programs has been made more representative of all segments of the extramural staff.

Acknowledgment

During the past year, the staff of the extramural programs has been working under numerous strains relating to reorganization, personnel decreases, and grantee relationships resulting from budgetary decisions. Despite this, the extramural staff, as administrators of the largest portion of the Institute's budget, has functioned efficiently and expeditiously in enabling the Institute to support the categorical research areas of its responsibility. As the concepts of the extramural programs enlarge and change, new opportunities and programs will emerge in which staff initiative can be expressed. It is fully expected that the present era of uncertainties will be but prologue to another age of exciting new developments keeping pace with the inherent vitality of research activities.

OFFICE OF THE ASSOCIATE DIRECTOR
FOR
DIGESTIVE DISEASES AND NUTRITION

ASSOCIATE DIRECTOR'S REPORT

Establishment of the Office of the Associate Director for Digestive Diseases and Nutrition

The Office of the Associate Director for Digestive Diseases and Nutrition was established in association with the passage of Public Law 92-305 in May 1972 (which changed the name of the Institute to the National Institute of Arthritis, Metabolism, and Digestive Diseases, designated that there be an Associate Director for Digestive Diseases, and established a committee of the NAMDD Advisory Council to advise the Director of the Institute on activities concerning digestive diseases); actions by the Senate Committee on Appropriations; and approval of the plans for reorganization of the NIAMDD Extramural Programs by the Office of the Director, NIH, in September 1972. The reorganization created, in addition to the Office of the Associate Director, the Digestive Diseases and Nutrition (DD-N) "cluster," which encompasses the Digestive Diseases and Nutrition Programs. Personnel in the cluster include the Associate Director, Digestive Diseases and Nutrition Program Directors and Assistant Program Directors, Special Assistant to the Associate Director, and four supporting staff.

The activities of the DD-N cluster can be characterized as both independent and cooperative. The cluster collaborates extensively with the Associate Director for Extramural Program Activities in operational and technical support activities, such as grant administrative and management services. Autonomy is maintained by the cluster in selection and administration of research, training, and fellowship grant programs and contract activities as well as in program analysis. The activities and new, innovative efforts of the cluster will be described below under each of the six functions outlined for the Office of the Associate Director. The grant and contract research supported by the DD-N Programs is described in the Program Reports.

Functions of the Office of the Associate Director

(1) Plans and directs a program of research grants and contracts, and training and fellowship awards for digestive diseases and nutrition to insure maximum utilization of available resources to attain program objectives;

The DD-N Programs obtained approval for and announced their intention to support a number of Digestive Diseases and Nutrition Centers. The objectives of the Centers are to create environments for the development of new and multidisciplinary research approaches to human digestive and nutritional diseases and for the acquisition of new knowledge essential for improved diagnosis, treatment and prevention of these diseases. The research efforts will include fundamental and basic aspects and will have a major emphasis on human diseases. Initially, Centers devoted to investigations of obesity, peptic ulcer, or hepatitis and cirrhosis will be supported.

The DD Program continued contract support of a controlled cooperative clinical trial on treatment of Crohn's Disease and initiated a contract for the planning of a cooperative clinical trial on the use of bile acid feeding for the dissolution of gallstones.

> (2) Develops, for presentation to the National Advisory Council, recommendations as to the modulating effect of categorical needs, desires, and demands on the priority judgments of scientific merit of grant applications made by the Initial Review Groups;

The Digestive Diseases and Nutrition Committee of the NAMDD Advisory Council was established in October 1972. The membership includes the gastroenterologists, nutritionists, and one public member of the Advisory Council, who will give advice in both program areas. The activities of the committee involved consideration of research and training grant applications presented to Council by the DD-N Programs, review and discussion of cluster activities, such as evaluation efforts, innovative efforts, program direction, etc., and consideration of contract proposals with preparation of a recommendation to be made to the Institute Director. At the full Council meeting, a summary regarding the Committee's recommendations or other comments on grant applications was made for the Council's deliberation. The Committee was found to function very satisfactorily in both advising the cluster and Director and in streamlining the Council presentation.

> (3) Prepares analyses of national research efforts to assist advisory groups in recommending new and/or continuing program emphases;
>
> (4) Maintains surveillance over developments in designated categorical areas and assesses need for research into the causes, diagnosis, prevention, and treatment of digestive diseases and nutrition and for training related thereto;
>
> (5) Develops and manages an information system, covering research developments and identifying areas where additional research and training efforts are required, for research scientists and other interested groups;

The DD Program has initiated an evaluation of the future needs of digestive diseases research. The purpose of the evaluation effort is to identify promising and important areas where too little effort is being expended and which could be augmented by deliberate initiatives taken by the Institute. Eleven areas of digestive diseases were defined. A Work Group was established for each area with the responsibility of preparing a series of papers that will define and justify the needs in specific areas of research. It is planned that these papers will be reviewed and criticized by the Chairmen of the Work Groups and the NIAMDD staff with advice from other NIH Institute staff in an attempt to establish a priority among the suggestions presented. It is hoped that this effort will also identify areas of research that overlap with interests of other NIH Institutes to avoid duplication of support and to initiate collaborative planning and research efforts.

An effort has been initiated to evaluate the research in the field of nutrition. Twenty nutrition research experts have responded to a request to define the field of nutrition and identify the areas of nutrition research that need to be pursued. An analysis of these responses has resulted in an outline of nutrition research responsibilities within and without of the NIAMDD Nutrition Program. A cooperation with the Nutrition Programs of other Institutes, especially the NICHD and the NHLI, will be utilized in the further differentiation of the various Programs' research responsibilities and priorities. It is anticipated that this early analysis effort will be expanded to an evaluation of research needs similar to that for the area of digestive diseases.

In conjunction with the Institute's publication of Gastroenterology Abstracts and Citations, the DD Program has begun an exploratory effort to retrieve selected data from published research on digestive diseases. The data will serve as the basis of the Program's information system (clearinghouse) for research data and developments in the area of digestive diseases.

(6) Advises and participates with outside lay (voluntary health) and professional organizations in studying and analyzing the needs and requirements of digestive diseases and nutrition.

The Digestive Diseases Program has taken an active role in the Second Conference on Digestive Disease as a National Problem in cooperation with several professional and lay organizations. Participation included a review of the suggestions resulting from the evaluation of the future needs of digestive diseases research, assistance in an analysis of the requirements for training in the area of digestive diseases and in an assessment of Federal resources available for research and training.

Activities of the Nutrition Program have involved interactions with both Federal and outside organizations. The Program has continued to play an active role in the operation of the Malnutrition Panel of the U.S.-Japan Cooperative Medical Science Program. A cooperative effort with the Growth and Development Branch of the NICHD has resulted in a better definition of the guidelines for research supported by each Program. The Office of the Associate Director and the Nutrition Program have participated on the DHEW Nutrition Coordinating Committee, the Federal Interagency Communications in Nutrition Group, the Interagency Committee on Nutrition Education, and the Department of Agriculture Trace Element Group, in an effort to promote communication among Federal agencies that support nutrition research and training. In addition, discussions of the research needs in the area of parenteral nutrition have taken place with the Parenteral Nutrition Committee of the Council on Foods and Nutrition of the AMA.

05 - DIGESTIVE DISEASES PROGRAM AREA

The Digestive Diseases Program (formerly Gastroenterology) encompasses research on organ systems associated with the gastrointestinal tract, including the salivary glands, esophagus, stomach, pancreas, liver, gallbladder, and small and large intestines. Research activity is concerned with the basic aspects of the physiology and biochemistry of disease entities. Efforts include studies of the cause, prevention, diagnosis and treatment of specific diseases such as esophagitis, peptic ulcer, pancreatitis, inflammatory bowel disease, malabsorption syndrome, gallstones, cirrhosis, and hepatic coma.

A. Progress in Research by Institute Grantees

1. Diseases of the Esophagus

a. Achalasia: The lower esophageal sphincter (LES) relaxes in response to swallowing to allow the passage of food. In patients with achalasia, this relaxation phase is impaired. However, the physiological mechanism of lower esophageal sphincter relaxation is not well understood, and two recent reports by NIAMDD grantees are directed toward the further understanding of this phenomenon. The elevated pressure in the LES is regulated by both neural and hormonal factors. Using the opossum, whose lower esophagus and LES is composed of smooth muscle fibers like that of man, one investigator found that prostaglandin E_1 causes a dose-dependent relaxation of the LES in the intact animal. Both theophylline and isoproterenol, agents that enhance cyclic AMP levels in many tissues, add to the action of prostaglandin E_1 to produce sphincter relaxation. The relaxant action is not modified by neural antagonists indicating an action directly on the sphincter muscle itself. Further studies of this type may provide the basis for drug treatment of achalasia.

2. Diseases of the Stomach and Duodenum

a. Gastric Ulcer: Substantial progress has occurred during the past few years in the understanding of a very common disease - gastric ulcer. New data suggests that a malfunction of the pyloric sphincter, which normally acts as a barrier which prevents reflux of duodenal contents into the stomach, may be the initiating factor in at least one type of gastric ulcer. Under normal conditions, the acid secreted into the stomach does not move back into the mucosa (gastric mucosal barrier) unless non-physiological amounts of acid are present: i.e. amounts almost twice as great as the normal stomach can possibly secrete. However, when bile salts (and other exogenous substances such as alcohol, salicylate, etc.) are present in the stomach, very small amounts of acid can cause a penetration of the mucosal barrier and effect the same extensive architectural and functional damage to the mucosa as occurs under extremely high acid conditions.

Studies of pyloric sphincter function reported by an NIAMDD grantee show that reflux from duodenum to stomach occurs readily in patients with gastric ulcer. In normals, the endogenous or exogenous administration of secretin and cholecystokinin (hormones that stimulate secretion of pancreatic juice and bile into the duodenum) cause an increase in pyloric sphincter pressure and thereby prevent reflux of duodenal contents into the stomach. In a small group of patients with gastric ulcer, this pyloric response to hormonal stimulation was entirely lacking. These duodenal contents, containing bile acids, then may permit the breaking of the gastric mucosal barrier at even low acid concentrations.

b. Duodenal Ulcer: In contrast to the results seen in gastric ulcer, where fasting serum gastrin levels seem to vary inversely with the state of gastric secretion (i.e. high gastrin - low acid secretion, low gastrin - high acid secretion), fasting serum gastrins of patients with duodenal ulcers are in the normal range in spite of extremely high acid secretion. The interesting new finding made by an NIAMDD grantee is that patients with duodenal ulcer with normal serum gastrin levels respond to feeding with an exaggerated gastrin release. Since duodenal ulcer is frequently characterized by a hypersecretory state, this phenomenon of more serum gastrin after a feeding stimulus may explain the anomaly of a normal fasting serum gastrin in spite of highly increased rates of gastric acid secretion. An increased sensitivity of duodenal ulcer patients to pentagastrin has also been reported by another grantee. This exaggerated responsiveness to the hormone also helps explain the finding of a hypersecretory state in the presence of normal levels of serum gastrin.

3. Inflammatory Diseases of the Liver

a. Exchange - Transfusion Therapy in Fulminant Hepatitis: A controlled trial of exchange - transfusion therapy for fulminant hepatic failure has been reported. It is known that the majority of patients with fulminant hepatitis and deep coma do not survive. Treatment with exchange transfusion has been advocated but not evaluated by appropriate controlled studies. Such a controlled trial has now been completed with twenty-eight patients with acute viral hepatitis. Although the number of patients randomized for exchange transfusion was small in number, it was reported that this mode of treatment did not increase the survival rate as compared to those patients who were not exchanged but treated conservatively. Although not conclusive, there was strong implication that the exchange transfusion treatment may have compromised the prognosis for this randomized and selected group of patients.

b. Treatment of Severe Chronic Active Liver Disease: A prospective, controlled, double blind, randomized trial of treatments for severe chronic active liver disease has been completed. It has been demonstrated that 20 mg of prednisone daily or a combination of 10 mg prednisone and 50 mg of azathioprine daily was superior to 100 mg of azathioprine daily or placebo. Prednisone or the combination of prednisone and azathioprine significantly improved (1) life expectancy; (2) resolution to normal of clinical, biochemical and immunochemical abnormalities; and (3) histological resolution from chronic active hepatitis to nonspecific hepatitis. By contrast, clinical and biochemical deterioration usually occurred with azathioprine or placebo. Side effects with prednisone were common with expected complications. Hepatotoxicity from use of azathioprine was not significantly different from the changes occurring during the natural course of the disease. Additional studies are required to determine the most effective mode of treatment, including dosages, duration of treatment, and incidence of relapse.

c. Endotoxin in Disorders of the Liver: Experimental laboratory data including preliminary clinical data have shown that endotoxin of intestinal origin is present in the blood in major non-specific disorders. The clinical data indicate that in a large percent of those with endotoxemia the liver is the site of the primary lesion or has been damaged by extra hepatic disorders. Other experimental studies have demonstrated that endotoxemia in the absence of sepsis is the result of liver injury or is due to endotoxin invading transmurally because the intestinal wall has been made more permeable by vasoactive agents released from a site of injury. It has been found that endotoxemia can be prevented by peritoneal lavage, by intra-aortic corticosteroids, by suppression of bacterial activity in the gut, or by a combination of these.

Additional studies will be undertaken to determine the incidence and significance of endotoxin in the blood and tissues of patients with major disorders of the liver with particular emphasis on the role of endotoxin on the development of major gastrointestinal bleeding and ulceration and the pathology associated with the later stages of liver failure.

4. Diseases of Gallbladder and Bile Passages

a. Medical Treatment of Gallstones: The finding of a diminished bile salt pool in patients with gallstones has led to the rationale of administering bile salts (chenodeoxycholic acid) to increase the bile salt pool toward normal. This approach is currently being tested therapeutically for dissolving gallstones. To date, in one study performed by an NIAMDD grantee, gallstone patients have been randomly allocated to three treatment groups; chenodeoxycholic acid, cholic acid, and placebo. After six months of treatment, gallbladder X-rays show the size of the gallstones are smaller in twelve of the nineteen subjects receiving a dose of 17-20 mg/kg/day of chenodeoxycholic acid, whereas none of the patients in either of the other two groups show any significant changes in stone size. These results have occurred with only minor or transient changes in liver function tests and therefore, the efficacy and safety of this treatment continues to look promising.

Theoretically, this approach may cause the cholesterol pool to become enlarged because bile acids will not be synthesized from the cholesterol, a potential hazard which has not in fact been demonstrated. Therefore, a more physiological approach might be desirable. The bile salt pool could be increased by increasing the synthesis of bile acids from cholesterol. This approach is now being studied in rhesus monkeys by a Boston grantee. Administration of phenobarbital, a drug which induces drug-metabolizing enzymes in the liver, on a chronic basis, increased the maximal rate of bile salt and phospholipid synthesis by about 30% without increasing cholesterol synthesis. These results were obtained at the relatively low dose of 5 mg/kg of phenobarbital, a dose which did not cause hypertrophy of the smooth endoplasmic reticulum of the liver. These investigators have now undertaken a clinical study to examine the effects of phenobarbital upon human hepatobiliary physiology and to determine whether a safe medical regimen of phenobarbital administration would be beneficial to patients with abnormal bile or with cholesterol gallstones.

A somewhat controversial finding is a report that hepatic bile composition improves after cholecystectomy. The composition of bile in patients with cholesterol gallstones was compared with that of patients without stones and found to be supersaturated with cholesterol relative to that of the controls. However, after recovery from surgery, these differences disappeared. Patients with cholesterol stones, after cholecystectomy, secreted bile as unsaturated with cholesterol as that of the controls. Confirmatory studies of these unexpected results are badly needed.

5. Colonic and Inflammatory Bowel Diseases

a. Lymphocytes and Inflammatory Bowel Disease: The function of the lymphocyte in inflammatory bowel disease has been the subject of recent investigations. Data has been obtained to elucidate the responsiveness of circulating lymphocytes to specific mitogens in patients with inflammatory bowel disease. Using phytohemagglutinin, fifty-eight patients with Crohn's disease, eighteen patients with ulcerative colitis, and twenty-one normal control subjects, it was found that lymphocyte responsiveness was impaired in approximately one-third of all the patients with inflammatory bowel disease, Crohn's disease and ulcerative colitis alike. This depressed lymphocyte responsiveness did not correlate with the anatomical extent, duration or activity of disease, nor with the presence or absence of lymphopenia. There is no evidence of a plasma factor or an indicated relationship to treatment with salicylazosulfapyridine. This impaired lymphocyte responsiveness may reflect an intrinsic defect of the immunocyte system which occurs equally in patients with Crohn's disease and ulcerative colitis.

Studies by other investigators have been completed to support the thesis that inflammatory bowel disease may be caused or perpetuated by immune mechanisms. It is known that the enterobacterial common antigen of Kunin is antigenic to colon cell membranes, and patients with inflammatory bowel disease were found to have elevated titers of humoral antibody to this antigen. The presence of cellular immunity to this antigen was demonstrated through the stimulation of lymphocytes to produce a migration-inhibition factor. Antigen-stimulated lymphocytes from patients with ulcerative colitis yielded marked migration-inhibition activity. Lymphocyte cultures from patients with regional enteritis were activated to a lesser degree by this antigen. Culture supernatants from lymphocytes of healthy volunteers or of patients with other intestinal diseases were inactive or minimally active.

b. Cell Renewal and Ulcerative Colitis: It has been postulated that ulcerative colitis is a mucosal disease of unknown etiology in which epithelial cell renewal may be abnormal. Data have been reported demonstrating both decreased and increased cell proliferation. NIH grant support effort has been recently completed demonstrating increased cell proliferation. This was accomplished by novel organ culture techniques. Epithelial cell proliferation and migration were studied in cultures of mucosal biopsies of the rectum from patients with ulcerative colitis and normal volunteers. More than twice as many epithelial cells in the zone of the rectal crypts were labeled in autoradiographs of ulcerative colitis biopsies than in normals. Also, many more labeled cells had migrated to the upper crypts and the absorptive surface in ulcerative colitis biopsies. Electron microscopy of the surface epithelium showed that the cytological abnormalities of freshly fixed biopsies persisted in the ulcerative colitis biopsies after twenty-four hours of culture. These studies clearly indicate that epithelial cell proliferation is increased and the migration of cells is accelerated in the rectal mucosa of patients with ulcerative colitis. In view of the well-recognized association of long-standing ulcerative colitis and colonic carcinoma, it was pointed out that in certain benign and malignant neoplastic lesions of the colon, upward displacement of the proliferative zone has been found.

B. Progress in Training

1. Research Training Grants: During this reporting year, ten training grant applications in gastroenterology (four new and six renewals) were reviewed, of which two new and six renewals were approved. The priorities were such that seven programs (two new and five renewal applications) were funded with a starting date of July 1, 1973. These supplement a non-competing group of twenty-five training grants giving a total of thirty-two active programs for July 1, 1973 - June 30, 1974. The total trainee spaces on these thirty-two grants are 111. Our estimate at this time is that all but two of the spaces will have trainees to whom commitments were made prior to the current phase-out plans.

2. Special Awards

a. The Clinical Investigator Award program added three new awards (of seven reviewed with four approved) to the five already being supported.

b. The Academic Career Development Award program remained at five active in spite of the fact that four additional applications were approved.

c. The Research Career Development Award program added four new awardees to the original group of seventeen for a total of twenty-one.

3. Fellowship Program: Of eighteen fellowship applications reviewed, nine were approved. However, only two were awarded while an additional four that had fundable priorities did not receive commitments before the phase-out plan. Total active trainees are fourteen.

Collaborative Research and Contracts

1. Current Contracts

a. National Cooperative Crohn's Disease Study: Nine major research centers across the country are now evaluating the therapeutic and prophylactic value of current drug treatments in regional enteritis. The National Cooperative Crohn's Disease Study is a controlled randomized clinical evaluation of three currently used drugs (prednisone, salicylazosulfapyridine, azathioprine) to determine their efficacy. This NIAMDD contract effort is programmed to extend to FY '76 and will expand by the addition of at least five more patient-contributing centers in FY '73.

b. Evaluation of Future Research Needs: The Digestive Diseases Program has initiated an effort with the aid of advice from scientists actively engaged in gastrointestinal research to evaluate the research needs in the area of digestive diseases. It is hoped that this activity will identify important areas of research where too little effort is being expended currently and will suggest research initiatives to be pursued.

2. Future Contracts

a. Gallstone Dissolution: The initiation of a contract effort by the NIAMDD to evaluate the efficacy and safety of chenodeoxycholic acid in dissolving cholesterol gallstones will be undertaken in FY '73. This effort will involve a one year planning phase followed by a four year clinical evaluation in which approximately 800 to 900 patients will be given the drug orally for a two year period.

b. Endoscopy Development: The development of more versatile fiber-optic endoscopic instruments which would allow controlled rapid advancement throughout the length of the small and large intestine and better diagnostic and therapeutic capabilities will be fostered by contract initiative. Other Institutes, in addition to the NIAMDD, have indicated potential specialized needs which would benefit from such research and these Institutes may contribute to a common funding effort.

3. Specialized Research Centers

a. Peptic Ulcer Centers: The NIAMDD is encouraging the coalescence of high quality research teams to concentrate their expertise on peptic ulcer disease in the hope of advancing our limited knowledge about the etiology and management of the disease.

b. Hepatitis and Cirrhosis Centers: It is planned to initiate centers to concentrate on multidisciplinary studies of these diseases of the liver in FY '74.

06 - NUTRITION PROGRAM AREA

The Nutrition Program of NIAMDD is primarily concerned with support of research having particular relevance to improvement of human health and longevity. Toward this end, it is involved with both basic and clinical research concerning the nature and the nutritional role of proteins and amino acids, carbohydrates, fats and fatty acids, vitamins, minerals and trace elements in normal and abnormal states of metabolism and disease. In most areas of research there has been encouraging progress, and in several areas notable new advances have been made.

Developments and Trends in Supported Research

1. Fat-Soluble Vitamins:

Last year noteworthy advances were recorded in identification of the metabolically active form of vitamin D, a hormone-like metabolite produced in the kidney through hydroxylation of vitamin D_3. This substance is of great clinical importance in the treatment of hypoparathyroidism and renal failure, but it has been possible to produce only minute amounts by tedious and expensive processes. Limited clinical trials have shown this sterol to be highly effective in treating abnormalities of calcium homeostasis in uremia of man. A hopeful solution to this problem lies in the recent report of successful chemical synthesis of this metabolite (1a-25-(OH)$_2$-D$_3$) with a demonstrated effectiveness five to ten times that of vitamin D_3 in curing rickets, elevating plasma phosphorus and increasing intestinal calcium absorption in experimental animals. There has thus been laid the basis for much more rapid and much less expensive production of this valuable therapeutic agent. Encouraging progress has been made in the elucidation of mechanisms involved in the synthesis and secretion by the liver of plasma proteins, lipoproteins, and the retinol-binding protein important in the transport and metabolism of vitamin A. Also of significance are recent observations clarifying confusing interrelationships between vitamin E and selenium, each of which has been known to compensate for deficiency of the other in certain biological processes such as protection of the erythrocyte against hemolysis. It now appears that vitamin E acts as a biological antioxidant within the red cell membrane, protecting it against oxidative damage, whereas selenium functions as a component of the enzyme glutathione peroxidase present in the stroma of the red cell. This seleno-enzyme, the first to be recognized in animal tissues, has many of the features of "factor 3" of Schwarz, which has never been adequately characterized. Interesting new developments relative to the biological role of selenium are anticipated.

2. Minerals and Trace Elements in Nutrition:

While modest support has been given to research on dietary iron, especially in relation to the worldwide problem of iron-deficiency anemia in pregnant women and children, there is anticipated an increase of research proposals related to this important problem. Research on zinc and chromium has been quite productive of new information. In addition to the demonstrated nutritional essentiality of tin, vanadium and fluorine during the past two years, two investigators have independently demonstrated that silicon must now be considered an essential dietary element in animal nutrition. It is considered to play a role in formation of mucopolysaccharides of connective tissue, and in initiation of the process of bone mineralization. New and interesting findings concerning the role of selenium as an integral component of a specific enzyme system have been referred to above.

Plans for Future Activities

1. Obesity Research : The Nutrition Program has identified the urgent need for the establishment and support of research centers for the study of obesity. As proposed, these centers would provide broad, coordinated, multidisciplinary approaches to the study of obesity involving parameters of the basic, clinical, and social sciences. Major areas of research that have been identified and that require study include the effect of dietary intake during postnatal life and of feeding patterns in childhood upon the later predisposition to obesity, the influence of glucose tolerance and insulin, effects of exercise and of psychological and emotional factors on food intake, and the impact of obesity upon the genesis and control of diabetes and upon the incidence of coronary artery diseases. Following an announcement in October 1972 requesting letters of intent from interested institutions, a total of 23 such letters were received. Scientific merit ratings on these have been requested from qualified outside reviewers. On the basis of their evaluations and subsequent review by Institute staff, a select few institutions will be invited to submit a formal research proposal. These, in turn will be subject to the usual NIH peer review process.

2. Future Direction of Nutrition Research: Special efforts have been initiated to obtain a critical analysis of current status and direction for future emphasis on nutrition research. Toward this objective, some twenty-five outstanding authorities in nutrition have been solicited for, and have submitted, independent appraisals and suggestions for most profitable areas of focus for nutrition research in the years ahead. The opinions expressed are in process of collation and summarization, for the further considerations of this group. This represents only the beginning of a more intensive evaluation study of the present status and future emphasis of research in the area of nutrition.

US-Japan Cooperative Medical Sciences Program (R22 grants)

A total of thirteen research grants (nine NIAMDD:four NICHD) will be supported by this program during Fiscal '73, as compared to eighteen during the previous year. This attrition is due largely to the fact that of ten grants terminating only two of six competing renewal applications received payable priorities. Further, only two out of ten new applications reviewed were accorded payable priorities. One of these was reclassified R01 and funded by NICHD, because of inadequacy of funds in the US-Japan program even though eight of the thirteen grants were funded at approximately 90% of the recommended level of support. It is anticipated that during Fiscal '74 funds for support of new and renewal applications, in addition to ten grants for which support is committed, will be marginally adequate. Since the initiation of this program approximately seven year ago, a total of thirty-two research projects have been supported (average duration of 3.4 years), and more than 200 publications acknowledging this support have appeared in the scientific literature. Moreover, a major objective of this program, the development and research support of the "Anemia and Malnutrition Research Center" in affiliation with the Chiang Mai Medical School at Chiang Mai, Thailand, reached an important high point with the official dedication in January 1973 of a new research building provided through combined support of the Thai government and the Rockefeller Foundation.

Fellowships: Support has been given to nine fellowships (five postdoctoral and four special) as compared to eight, ten, and six during FY'70, FY '71, and FY '72. The four Clinical Investigator Awards and one Academic Career Development Award in nutrition activated during Fiscal 1972 were augmented by an additional ACDA in Fiscal 1973.

Training Grants: Training Grants, which provided support for nineteen predoctoral and nineteen postdoctoral trainees were reduced in number from eleven to eight. Although two new training grants received approval in a payable category, at November 1972 Council, subsequent events precluded their activation.

Official Duties: The Nutrition Program Director has continued to serve as Secretary of the Malnutrition Panel of the US-Japan CMSP. Subsequent to Joint Malnutrition Panel meetings held in Kyoto, Japan, November [illegible], 1972, he was authorized to visit and confer with three grantee investigators and their associates in Bangkok, and also to visit the Anemia and Malnutrition Research Center in Chiang Mai, Thailand. Each of the visits was highly rewarding. During this year he has also served as a member of the Food and Nutrition Advisory Council of the Nutrition Foundation, and as a member of the Corporation of the Muscular Dystrophy Association of America. During the coming year he will again serve on the Scientific Advisory Committee of the latter organization, from which he had resigned a year ago.

01 - ARTHRITIS PROGRAM AREA

The Arthritis Program encompasses research activities covering a wide range of closely related subjects, such as the arthritides, connective tissue (collagen) diseases, joints and their contents, inflammation, immunology (specifically, autoimmune conditions), chemotherapy of arthritis and connective tissue diseases and skeletal muscles.

The three major forms of this disease are (1) rheumatoid arthritis, which afflicts about 5 million Americans and is the most crippling form of arthritis, (2) osteoarthritis, which affects approximately 16 million Americans and is generally associated with aging and degeneration of joint tissues, and (3) gout, which affects about 1 million Americans and most frequently affects the joints of the feet, especially the big toe.

Arthritis. Although research has been ongoing for a number of years, the etiology of rheumatoid arthritis is still unknown. Evidence strongly suggests microbial organisms as being a contributing factor in arthritis of animals up to and including subhuman primates, but not in man. Attempts to implicate viruses or virus-like agents as a possible cause of arthritis in animals and man have not been successful. Studies on the possible role of microbial organisms in arthritis are continuing.

Osteoarthritis. A grantee recently showed that repeated sudden impact to the foot in animals causes microcracks in the bones of the knee joint. Healing of the cracks with subsequent loss of resiliency of bone immediately below the articular cartilage initiated degeneration of the cartilage similar to that present in degenerative joint disease (osteoarthritis).

Gout. Research in gout is supported at a considerably lower level than the arthritides. This is due in part to the fact that gout can be treated with a greater chance of alleviating symptoms and it affects fewer individuals. The exact etiology of gout is unknown. Research is, therefore, continuing in an effort to elucidate possible causes. A number of recent reports have implicated the Hagerman factor, kinin systems, lysosomal products of polymorphonuclear granulocytes, or complement as being the cause of the inflammatory process in acute gouty arthritis. Additional research must be done to identify the responsible factor(s).

Connective Tissue. Researchers are slowly unravelling the defects in some of the hereditary diseases of connective tissue. For example, the activity of alpha-L-iduronidase has been found to be deficient in select tissue extract from patients with Hurler's syndrome but not in tissue extracts from patients with Hunter's or Sanfilippo's syndromes.

Other studies have concentrated on the biochemistry of collagen and, more specifically, on the enzymes of collagen, such as protocollagen lysine hydroxylase, proline hydroxylase, and their role in the structure and function of collagen.

Preliminary results on collagen secretion by connective tissue cells indicate that these cells secrete complex molecules into their environment as "procollagen" rather than collagen. Collagen formed from this "procollagen" is resistant to pepsin digestion and is probably a triple helical unit.

One grantee proposes that various collagens, procollagens, basement membranes, and related connective tissues may become strongly antigenic when modified by endogenous physical or chemical factors. He is, therefore, studying the antigenic determination of these tissues in an effort to verify this hypothesis.

Immunology. The autoimmune mechanism is still suspect as a factor in rheumatoid arthritis. No major developments have occurred in this area during the past year.

Clinical Trials. The Cooperating Clinic group has completed two clinical trials using an immunosuppressive drug (cyclophosphamide) either at a high or a low dose. The high dose level produced a marked decrease in clinical symptoms in the RA patient and the erosion of the joint as detected by X-ray appeared to have been arrested. The low dosage treatment produced few significant clinical changes and did not arrest joint erosion.

Systemic Lupus Erythematosus. Research on SLE in a dog colony appears to indicate a filterable infectious agent in the tissue of the diseased animals which can be transferred from one generation to the next. This finding may well be of major importance in understanding the etiology of SLE and development of methods for treatment or prevention.

Miscellaneous. Basic research of skeletal muscle is continuing. One grantee has established a tentative amino acid sequence of skeletal muscle actin of the rabbit based on cyanogen bromide peptides. Successful completion of this study will permit use of this sequence as a reference with which to compare sequences from other actins and to establish the relationship between various sources. Selective breaking of the sequence may also be possible, which would permit a wide variety of chemical modifications.

Training Grants. There were 27 active training grants in arthritis during this fiscal year, and no new grants were initiated during this period.

09 - ORTHOPEDICS PROGRAM AREA

The interests of the Orthopedics Program Area include clinical and basic science studies of the skeleton in diseased and normal conditions. Basic studies include structure and function of bone at cellular and subcellular levels. Various techniques such as X-ray diffraction, crystallography, electron and scanning microscopy, nuclear magnetic resonance, electron probe analysis, optical rotary dispersion, and circular dichroism, have been utilized in the study of bone.

Artificial Joints. Studies are underway to determine the best possible materials for fabrication of joints that when implanted will be non-toxic, non-antigenic, and exhibit little wear or corrosion, so that the joint need not be replaced and will remain securely attached to the skeletal or muscular system *in vivo*. The artificial hip is probably the most advanced of the joints under study. Major faults still exist in the choice of materials used to fabricate the joint and in methods of attachment. Researchers are presently working with carbon reinforced plastics and ceramics, TRIP steel, and various metals. Porous metals and metals coated with porous ceramics or plastics are being used in an effort to obtain skeletal tissue implant attachment through growth of tissue into the pores of the implant. Retaining the implant in an absolute stationary state until sufficient ingrowth has occurred to stabilize the joint presents a problem. In those cases, however, where ingrowth has occurred this method compares favorably to methacrylate bonding.

The material presently showing the most promise for joint implants from the consideration of wear characteristics is a combined vitallium-ultrahigh molecular weight polyethylene. Carbon reinforced-ultrahigh molecular weight polyethylene also exhibits a high degree of wear resistance *in vitro*. *In vivo* testing for toxicity and immune reactions is underway.

The development of other artificial joints has not progressed as rapidly as has the hip joint although somewhat primitive knee and finger joints are now available and in use.

Fractures. One grantee has initiated a research program to define a reproducible long bone fracture in an effort to study bone healing rates under different methods of treatment including delayed fixation of the fracture. Perfecting a reproducible fracture will represent a major step in the study of bone healing and callus formation.

Electrical Current. Enhancement of bone healing through the use of electrical current in the microamperes range is being pursued in several laboratories. An electrode is applied on or near the fracture or non-union site. Current is applied at varying amperages and voltages over several days. Histological and radiological examination, post treatment, have shown increased cellular activity and rate of bone deposition around the electrode bone-contact indicating that properly applied electrical current does increase healing rate of bone.

Immunology. Bone immunology is still in an embryonic stage. It is becoming increasingly evident that additional studies are needed in this field, especially as bone implants increase in number. Support for this area is not large at the present time but should increase as researchers in bone become experienced in immunology.

Osteoporosis. A five-year study of osteoporosis is nearing its termination. A total of 165 females age 38-44 were in the group. Final analysis of the data is underway.

Articular Cartilage. One grantee is attempting to perfect a method of preserving and storing articular cartilage so that it can be transplanted while still in a viable state. Synthetic cartilages have not been successful and the need for viable transplantable cartilage is evident.

Scoliosis. Scoliosis is being approached via pathophysiological evaluation of the disease, including pulmonary function tests prior to and after corrective surgery, two-plane radiographic configuration evaluation of the spine, oxygen consumption for patients using the Milwaukee brace, reflex balance and motor coordination testing, cardiac output, and monitoring of the neuromuscular and musculoskeletal systems.

Computer generated force analogs of the human spine have been constructed through acquisition of data from living and cadaveric scoliotics. Models will be built from these data; revision will occur as new data become available.

Endocrinology. The relationship of growth hormone to longitudinal skeletal growth is being studied by one grantee. These studies include the nature and source of the sulfation factor (somatomedin) and the biological interactions between it and other hormones. Assay of this factor is being attempted through use of articular chondrocyte cell cultures. A recently discovered factor secreted by lymphocytes appears to have bone resorbing activity and is being studied along with vitamin D metabolites, prostaglandins and their interaction with bone.

Implant Materials Review. For the past year a materials engineer (Presidential Intern, National Bureau of Standards) has been reviewing the current status of implant materials use and associated materials research. Information for this subject was obtained through meetings, workshops, conferences, visiting research laboratories, and through extensive library research. A current bibliography and cross-referenced information system have been developed. Interim reports have detailed progress. A concluding final report will be submitted.

Training Grants. Two new training programs were funded in orthopedics during fiscal year 72, bringing the number of active grants to eighteen.

02 - DERMATOLOGY PROGRAM AREA

Targeted research within the Dermatology Program Area is directed to the search for and the development of a safe, effective topical agent for the control of psoriasis. A dual approach to finding this agent is currently being planned. First, as indicated in the 1972 Annual Report, a proposal has been submitted jointly to the National Cancer Institute and NIAMDD to develop a cooperative screening program of chemotherapeutic drugs for the topical therapy of psoriasis. Current plans consist of establishing a study with minimal investment to determine whether the empirical search for a cancer chemotherapeutic type of drug can be used effectively and safely for topical treatment of psoriasis. This study would be supported by means of a contract with four or five departments of dermatology, one of those departments acting as a coordinative center. To properly monitor the study and the results, there would be a scientific director, pharmacologist, and biostatistician. The second approach will investigate further the possibility that epidermal cell proliferation may be regulated through the cyclic AMP system. It is now apparent that in some cells, cyclic AMP acts as the "on switch" and in others as the "off switch", and that the same is true of the other cell messenger, cyclic GMP. It is not safe to take cyclic AMP internally but with this whole new field of scientific inquiry which has recently opened up, it appears that further research may well turn up agents safer and more effective than those now used to control psoriasis. A more physiological agent used to control the disease would hopefully have less toxic effects on the normal cells of the body when used systemically to control a specific population of cells that are undergoing excessive proliferation. A small workshop on the role of cyclic AMP on growth control in epithelial proliferation and differentiation to be supported by the NIAMDD in the fall of 1973 is being planned. Recommendations from that workshop will be utilized by NIAMDD staff and its advisors to establish additional directions of investigation.

Grant support from the Dermatology Program Area is directed mainly by providing continuous basic research at the cellular and biochemical level so that the proper type of knowledge can be established. This is required in many instances in order to further understand and be able to treat certain cutaneous disorders. One grantee is studying the chemistry of keratins and their precursors. His work characterizes in detail the structural and chemical properties of the main components of protective epidermal cells and will serve as reference standards for the evaluation of changes in pathologic cells such as are formed during wound repair and in psoriatic patients. The work on prekeratin provides information for the first time on molecular organization of the fibrous proteins (alpha-protein) forming the main structural component (60 to 80 Angstrom filaments) of most keratinizing tissues. Other studies on the biosynthesis of keratin have allowed investigators to pursue clinical studies of ichthyosis and other hereditary disorders of keratinization. Using mixtures of propylene glycol in water as a keratolytic agent, investigators have confirmed their initial impressions that this is a very effective form of therapy in the range of 40 to 60 percent in water. Additional studies involve the use of vitamin A acid (retinoic acid) for hyperkeratotic disorders of the skin.

The role and effect of hormones on the sebaceous gland in man continues to be studied. Current investigation includes (1) utilization of the sebaceous glands for assay of androgens and antiandrogens, (2) measurement of sebaceous gland activity in endocrine diseases for base line activity and changes as the result of treatment, and (3) measurement of androgenic steroids and their metabolism *in vitro* by the skin and sebaceous glands. The overall goal of these studies is the ultimate development of a modality of hormonal therapy reducing the activity of the sebaceous glands which are necessary for the development of acne. In addition, particular emphasis is being placed on the prevention of enzymatic breakdown of the lipid formed by the sebaceous glands.

Investigators are also studying the role that prostaglandin inhibitors might play in sebaceous gland control, the dietary control of sebaceous gland function and the role of microorganisms in the genesis of free fatty acids.

Studies of granuloma-type sensitivity are contributing significantly to the understanding of the histogenesis of epithelioid cell granulomas. In addition, studies with "transfer factor" have demonstrated that granulomatous hypersensitivity can be transferred as well as delayed hypersensitivity. Information obtained from these studies may offer a basis to understand not only experimental granulomas but also other granulomatous disease, such as sarcoidosis, tuberculosis, and leprosy.

Biochemical studies of altered dermal tissue have revealed new information on the pathologic process in scleroderma. It appears that induration of the skin in scleroderma is primarily due to the replacement of subcutaneous tissues by newly synthesized connective tissue. In addition, it appears that this replacement phenomenon occurs in all forms of scleroderma.

Systemic corticosteroids are widely used in therapeutic medicine today. It has been demonstrated previously that healing is delayed in experimental wounds of subjects on long-term corticosteroid therapy. Our grantees have observed electron microscopic changes in these wounds not heretofore recorded. It appears that the characteristic spiral configuration of ribosomes on endoplasmic reticulum membranes of fibroblasts in normal wounds occurs with less frequency in the steroid-treated wounds. Studies are continuing to outline and define these structural abnormalities.

Through the support of NIAMDD the Dermatology Program Area has observed over the years the advances made in the diagnosis and treatment of pemphigus vulgaris, an extremely lethal disease. In addition, the immunofluorescent technology that has been developed in the study of pemphigus now has wideranging applicability and is being used by many scientists to diagnose, evaluate, and monitor treatment of the burgeoning collection of autoimmune diseases. Now that fluorescent techniques have become established the goals of our grantees are to characterize the biochemical nature of the pemphigus antigen, to elaborate a well defined reproducible method for passively transferring pemphigus and possibly also bullous pemphigoid to monkeys with the aid of pemphigus antibodies, to evolve methods for detecting trace amounts of free pemphigus antibodies in the circulation with a view toward finding methods to remove or destroy them, and be able ultimately to provide complete eradication of this disease in human beings.

An exciting area of basic research within dermatology involving mechanisms of connective tissue degradation is evolving. Very recent studies from these laboratories have described a specific human skin collagenase which was isolated and partially characterized from culture medium in which living skin explants were grown. Current objectives of these investigators are to obtain detailed understanding of the mode of action of animal collagenases and of the human collagenase in the mechanisms involved for controlling their biosynthesis and subsequent activity. Utilizing this information one will be able to apply this to the study of wound healing and uterine resorption, and learn more of the pathogenesis of certain skin diseases and rheumatoid arthritis as well as the relationship of collagenase to both cutaneous and uterine neoplasia.

In the 1972 Annual Report there is reference to the effectiveness of carotenoid therapy in cases of photosensitive porphyria. To date, 51 patients with erythropoietic protoporphyria have been either completely cleared of their disease and totally protected in the sunlight by treatment with carotene or have markedly improved and can lead almost normal lives. Thus far the photoprotective efficacy has proven itself and there is no evidence of toxicity from ingestion of beta carotene.

Finally, to back up the empirical search for effective topical agents in psoriasis, research is being pursued at the cellular level to learn more of the basic defect that occurs in psoriasis. One grantee has reported that the dermis effects great changes in the behavior of the epidermis. It can thus be argued that the major changes observed in cytokinetics in psoriasis may reflect dermal influence. Fibroblasts from patients with psoriasis are being cultivated for periods up to one month in order to determine whether this dermal influence is significant. Working from the therapeutic end of the spectrum, one investigator is studying the percutaneous absorption of some of the antimetabolites. Investigators have been able to gather information with regard to penetration of methotrexate through human skin and the hairless mouse skin. Although methotrexate has not been effective topically for the treatment of psoriasis, the experimental systems indicate that there is penetration of methotrexate through skin. They are also attempting to work out the penetration of 5-fluorouracil through human skin. In addition, they are investigating a number of other metabolites for their effects on the mitotic index in normal human skin when applied topically and under occlusion.

10 - PHYSICAL BIOLOGY AND RELATED AREAS PROGRAM AREA

As noted in previous reports, the investigations supported in this program are characterized by their wide scope and diversity. They include the application of newly developed physicochemical methods to the study of living systems and molecules of interest, and syntheses of these molecules and their analogs.

One noteworthy contribution has been to devise a method for fixing and embedding preparations of subcellular particles for quantitative electron microscopy. In contrast to sedimentation or filtration techniques, the particles are as uniformly dispersed in the final polymerized plastic as they had been in their original aqueous suspension. First efforts in this direction involving the use of water soluble embedding media and polymerization in the cold with ultra-violet light had limited success, as the particles were difficult to section and preserve. A successful technique resulted when subcellular particles were fixed in suspension and then centrifuged forming a very friable pellet, which was completely redispersible with adequate handling.

The revised method works equally well for microsome fractions, mitochondria and even crude liver homogenates; the suspensions are quite uniform and there is no tendency for settling or clumping during polymerization. Electron microscopic observation of the dispersed particle suspensions shows that mitochondria are intact and ribosomes are still attached to the rough surfaced microsomes. Their appearance is indistinguishable from particles prepared by the conventional pellet technique or the ultrafiltration method of Baudhuin et al (1967). This technique opens the possibility for relating chemical and enzyme assays directly to the number, volume and surface areas of specific particles determined by morphometric methods.

The wonder of nature is readily appreciated in the microcosm of structural proteins. Here, by subtle chemical variations, materials possessing high tensile strength (tendon), or elasticity (elastin), or physical integrity (skin epidermis and bone) have evolved in the collagen family. Protein chemists and physicists have called on many tools to study the molecular structures of these materials from the primary to the quaternary levels. However, as the function of the molecule departs from a purely chemical one (i.e. enzymes and nucleic acids), the morphological aspects of the system become increasingly important to account for its physical and mechanical properties. It is these latter areas that our supported programs seek to investigate. In particular, one tries to understand how the triple helices in a given material are assembled into a supermolecular unit to give its unique set of characteristics and how collagen together with mucopolysaccharides and apatite create the main supporting frameworks for living species.

An investigator has demonstrated that native collagen possesses a non-helical, non-collagenous terminus called telopeptide. It has been shown to be the determinant for immunological activity and polymerizability. It is interesting to speculate whether the telopeptides are also determinants for physical mechanical properties. On the basis of species specific antigenicity, collagens, which have their telopeptides cleaved by a proteolytic enzyme, are a natural choice as a material for implantation surgery. But the absence of telopeptides could significantly reduce the desirable properties of the native collagen. Perhaps grafting of an inert non-collagenous material to enzyme treated collagen may give the best of both worlds. In this year, the plan is to exhaustively compare the mechanical and physical properties of acid soluble collagen, which is native, and enzyme soluble collagens. The techniques used will be small angle light scattering birefringence, dynamic birefringence and dynamic viscoelasticity.

The 1972 Novel Prize in medicine has been awarded jointly to one of our grantees, Professor Gerald Edelman of the Rockefeller University, New York and to Professor Rodney Porter, Whitley Professor of Biochemistry at the University of Oxford, England.

The recognition of the work of these biologists on the determination of the structure of the antibody molecule comes twelve years after their epic studies were first published. At the time Professor Porter was on the staff of the National Ministry of Health Laboratories at Mill Hill, London and Dr. Edelman was then, as now, at the Rockefeller University.

The complementary work of Porter and Edelman culminated in a complete description of the structure of the human gamma globulin molecule. Porter, using rabbit anti-ovalbumin, was first able to divide the molecule into three functional parts: two identical fragments which bind antigen and must thus contain the sites involved in their recognition, and one fragment which is invariant.

Edelman then found a different way of splitting the antibody molecule by reducing it with mercaptoethanol in strong urea, showing that it was composed of polypeptide chains bound together by disulphide bonds. This led Porter to refine Edelman's technique and resulted in the confirmation of the four-chain model for gamma globulin: two heavy chains and two light chains, each with variable binding sites at one end and joined by disulphide bonds at the invariant regions at the other end.

Porter's technique made possible work in both laboratories on determining the amino-acid sequence of the variable portions of the heavy chains and showing that the variations were similar to those which had already been found in the light chains, suggesting that the two were complementary. The further development in Porter's laboratory of a method of splitting the heavy chain further, into more manageable fragments, finally enabled Edelman to sequence an entire immunoglobulin molecule for the first time.

In another area of research activity, the basic philosophy underlying analog studies has been expressed by J. H. Wang, who has studied biological analog systems over many years:

> "While experiments on biological systems continue their admirable progress,...because of their relative simplicity, relevant synthetic models sometimes allow us to identify the crucial factors among a large number of possible causes... and reach the core of the more complicated biological problems much sooner."

The systems studied by one of our supported investigators and the focus of interest will be rather different from Wang. Wang has tended to concentrate on systems, not necessarily porphyrins, that simulate biochemical reactions. This work concentrates on synthetic porphyrin systems with electronic structures similar to the biological systems and studies their electronic spectra and electron transfer properties.

The mechanism for the functioning of iron porphyrin in oxygen binding and in biological redox is not fully understood. Similarly the mechanism by which photoexcited chlorophyll produces an electron transfer leading to a free radical rearrangement is not understood, although it is known that an iron porphyrin plays a role as an electron donor in this process. Finally, a system that efficiently converts solar photons directly to electric current through cheap solar cells has yet to be discovered. Such a system could conceivably provide a solution to man's long-term source of energy in a totally nonpolluting way.

03 - DIABETES PROGRAM AREA

The major aims of the diabetes program are to define the disease fully in terms of its causes and many complications, and to find methods for optimal treatment or prevention. To prevent or control diabetes, two goals must be achieved. First, better methods must be established for detecting the disease as early as possible, and even for predicting its future occurrence in certain patients. Second, the genetic and metabolic causes of the disease, and the metabolic changes which lead to its serious complications (e.g., microangiopathy) must be understood in molecular terms so that rational methods of treatment and prevention can replace the present empirical and inadequate approaches.

There are at present four to five million known patients with diabetes in the United States, and an estimated five million other persons are potential diabetics -- persons who may develop the disease during their lives. Rated as the seventh leading cause of death from disease, the true toll of diabetes is considerably higher since its vascular complications contribute heavily to the cardiac and cerebrovascular death statistics. Added to this are the many lost man-hours caused by complications of the disease -- retinopathy and blindness, kidney malfunctioning, cardiovascular problems, increased susceptibility to infection, and neuropathy -- and an estimated $2 billion which diabetics spend for medical care each year in the United States alone.

During FY 1973, the number of approved, on-going research projects was reduced by the limited availability of funds from 110 to 107. An even greater reduction would have occurred were it not for bloc renewal of a 13-project collaborative study known as the University Group Diabetes Program. All of the 73 continuation projects were funded, many of them at less than the recommended amounts. Only 26 of 35 approved renewal applications could be funded. Of 44 approved new proposals only 8 will be funded. The 107 research projects constitute the most direct approach of the Institute to the many problems associated with diabetes. A wide range of objectives, approaches and interests are reflected in the research projects; these have been classified for planning purposes into the four major groups described below.

Etiology, Pathogenesis and Treatment

Forty of the projects are concerned with the causes and diagnosis of diabetes, the complications of the disease, and the efficacy of current or potential treatments. These projects place emphasis on the study of diabetes in human subjects to complement research in experimental animals. Included are studies of the role of inheritance and diet, changes in metabolism and enzyme concentrations in the diabetic state, characterization of the complications which follow initial development of the disease, study of the mechanism by which known diabetogenic agents cause or stimulate the disease, and testing of current and potentially new methods of treatment. Only a few of the projects involve detailed studies of the development of diabetic vascular complications, and much more emphasis on this subject is needed.

About one third of these projects are designed to determine the extent to which hypoglycemic agents (insulin and synthetic drugs), or carefully selected diets, control the complications developed in the diabetic state (including diabetic retinopathy and neuropathy, glomerulosclerosis, microangiopathy), and effects on longevity.

Recent progress in this group of projects is exemplified by work on capillary basement membranes. Many investigators have demonstrated that, in general, capillary basement membranes are thicker in diabetics than in nondiabetics, but few investigators have attempted to relate this phenomenon to the duration of known diabetes, to the degree of carbohydrate intolerance, or to the extent of insulin deficiency in the involved individuals. Such data are of utmost importance in establishing a possible causal relationship and in determining the orientation of research on the pathogenesis of diabetic microangiopathy. Recent observations on diabetics suggest that basement membrane thickness is probably normal in most subjects prior to the onset of carbohydrate intolerance, and that both the magnitude and incidence of basement membrane thickening increase with longer duration of carbohydrate intolerance. It is more common in diabetics over 50 years of age than in those under 50 years of age, and the incidence increases to 90% in diabetics with retinopathy and/or nephropathy, and to 93% in those who have had the disease for 20 years or more. Continuation of these studies, and the development of simpler methods for measuring the basement membrane, should yield a reliable means of assessing the effects of new treatments on the complications of diabetes.

Secretion and Activity of Insulin and Related Hormones

Thirty-three of the projects involve studies related to insulin secretion and activity under a variety of circumstances in normal and diabetic patients and in experimental animals. These include the histology and physiology of the pancreas, insulin biosynthesis in isolated cells and in secretion granules of the islets of Langerhans and the many factors regulating this synthesis, abnormalities in insulin secretion in the diabetic patient or animal, improved techniques for measuring insulin activity, antibodies and inhibitors which may block insulin action, factors which affect the degradation of insulin, and other hormones which may oppose or supplement the action of insulin.

Transplantation of insulin-producing cells is an area of current interest in this class of projects. It was shown previously that when normally functioning pancreas was transplanted into severely diabetic patients, the transplanted organs produced sufficient insulin to control blood sugar in the recipients for a limited period. Eventually, however, the transplanted pancreas suffered tissue rejection through immune reactions of the host patients. More recently, workers at Washington University, St. Louis, have demonstrated that pancreatic beta cells, transplanted into the thigh muscle or the peritoneal cavity of diabetic rats, survived and produced sufficient insulin to significantly lower blood and urine sugar levels in the recipient animals. These studies -- transplantation of pancreatic organs and cells -- must be continued in view of the great potential for benefit to the diabetic patient.

A related activity is development of a so-called "artificial pancreas," which consists of a sensitive monitor of blood glucose and a pump to release insulin into the blood in response to a signal from the glucose monitor. At least two laboratories appear to be making progress toward development of a unit, including the monitor, the pump and a several-month supply of insulin, which can be "transplanted" under the skin of the diabetic. Continuance of this work is important for its potentially great benefit to the diabetic, and also because the unit may assist in determining whether rigorous control of blood sugar in the diabetic will indeed prevent development of the morbid complications of diabetes.

Mechanism of Action of Insulin

Twenty-eight of the projects are primarily concerned with the precise biological functions of insulin and the mechanisms by which these functions are fulfilled. Subjects under study include the relationship of the structure of insulin to its activity, factors which modify insulin action, the role of other hormones in regulating insulin activity, the mechanism by which insulin controls blood sugar levels, and the effects of insulin on the metabolism and transport of carbohydrates, lipids and proteins. These studies are fundamental in nature and commonly involve investigation at the molecular level.

Recent progress in this area offers encouragement for a better understanding of diabetes and for concrete improvement in the treatment of the diabetic patient. NIAMDD grantees have provided good evidence that insulin acts upon individual body cells primarily by binding to specific receptor sites on cell membranes, and that the multiple effects of insulin, e.g., increased glucose transport into the cell, decreased lipolysis, increased glycogen synthesis, depend upon one or more "second messengers." Possible candidates for these second messengers are certain polyamines, such as spermine and spermidine, which have been found to mimic some of the actions of insulin at a point subsequent to the insulin-receptor interaction. Another possible second messenger for certain insulin effects is cyclic AMP, since the cellular concentration of this compound is markedly decreased by insulin under some conditions. Continuance of such fundamental studies can be expected to reveal the mechanisms by which insulin exerts its multifarious effects on cellular processes, and may permit development of rational and effective treatments for diabetic patients.

Carbohydrate and Lipid Metabolism

Six of the projects are best characterized as comparisons of carbohydrate and lipid metabolism in diabetic vs normal individuals or animals. Subjects under investigation include pathways for catabolism of carbohydrates and lipids, gluconeogenesis, synthesis and interconversions of fatty acids and lipids, storage and mobilization of lipids in adipose tissues, and the control of such processes by hormones, particularly insulin. Such studies are, of course, essential for a full understanding of the changes which occur in the diabetic state.

An example of work in this area is a project which seeks to determine the location, nature and factors regulating various pathways of carbohydrate metabolism in muscle and to establish their physiological significance, including the effects of insulin. Such studies are important both to a better understanding of insulin action and to development of new approaches to management of the diabetic patient.

Plans for Future Activities

Opportunities mentioned above, and others omitted for the sake of brevity, will be pursued as vigorously as available funds permit. In addition, a collaborative multiclinic study of the long-term effects of current methods of treating adult-onset diabetes will be continued at least until August 31, 1975. This study, now in its thirteenth year, has suggested that long-term treatment with two of the oral hypoglycemic drugs, tolbutamide and phenformin, increases the likelihood of death of the diabetic from cardiovascular causes. Use of the oral drugs in this study has been discontinued, but the surviving patients are still being followed while treated with diet alone or diet plus insulin. In other patient groups, two insulin treatments have begun to show decreased mortality in comparison to the control group receiving placebo tablets. The differences are not yet significant, but the study must be continued in view of the obvious implications for treatment of diabetics.

Although research on basic mechanisms, such as those discussed above, can be expected eventually to benefit the "juvenile" as well as the adult-onset diabetic, the Institute currently supports too little research bearing directly on the problems of the juvenile diabetic. Reasons for this are the very delicate ethical problems encountered in using younger patients in controlled studies. Despite these problems, the Institute will investigate means by which research efforts associated with the unique complications of juvenile diabetes can be increased.

Finally, the Institute hopes to initiate support of a number of Diabetes-Endocrinology Research Centers, and has already received 14 applications for these. During the next year or two, only centers in which the research emphasis is in the area of diabetes will be supported. Center grants will be limited to institutions where there is substantial high quality ongoing research in diabetes, and will be used to foster futher development of these research efforts. These centers are conceived as providing core resources and services to accomplish research goals that would not otherwise be achieveable. Although these grants are not intended to give direct support for established research projects, funds to initiate projects by new investigators and for exploratory or feasibility studies may be furnished. It is anticipated that these centers will substantially increase the research output in selected institutions.

Hormones Affecting Mineral Metabolism

Cooperation between NIH intramural scientists, university grantees, and a pharmaceutical company has paid off in the form of determining the structure of the biologically active portion of the human parathyroid hormone. This hormone has 84 amino acids, the first 34 of which form the biologically active portion. AM grantees Claude Arnaud and Glen Sizemore from the Mayo Clinic collected parathyroid tumors over a two-year period until they had enough material for extraction. Final purification and sequencing was done at NIH by intramural scientists. Finally the hormone was synthesized by a team of scientists at Ciba-Geigy in Basel, Switzerland. The active synthetic hormone can be used clinically, and especially in developing radioimmunoassay methods to help in the diagnosis of parathyroid disease. Dr. Arnaud and associates continue active research on the other hormone affecting calcium metabolism, thyrocalcitonin. His ultimate aim is treatment and/or prevention of such diseases as osteoporosis.

Dr. Eric Reiss, University of Miami, has shown that fragments of parathyroid hormone can be detected by radioimmunoassay. The significance of these fragments is not clear since it is unknown if fragments are secreted from the parathyroid gland or are generated as a result of metabolism of the hormone in peripheral tissues. Studies are underway which will help answer this question. Until answers are obtained, the radioimmunoassay of parathyroid hormone will be difficult to interpret.

Radioimmunoassay for calcitonin has proved to be a valuable tool in a way not suspected. Since the other hormone controlling calcium metabolism is produced in the thyroid, assay methods in which the level of calcitonin is determined following infusion of calcium tend to show up overproduction of the hormone. If this is present, it has been correlated with thyroid medullary carcinoma, early detection of which can be successfully treated. Dr. Armen Tashjian of Harvard has been pursuing studies of this sort.

Dr. David Cohn has produced evidence showing that parathyroid hormone may be originally manufactured as a "prohormone" of larger molecular weight than the circulating hormone. He plans now studies to determine the biological significance of this.

Steroids

Dr. Gert Jacobsohn is studying the role of red blood cells in assisting in the transport of steroids from the site of manufacture to their site of action in the body. He is also interested in the manner in which relatively inactive steroids with a ketone group at the 17 position are changed to active hormones with a hydroxyl group in position 17. An enzyme, which is being characterized, appears to be involved in this transformation.

Thyroid Hormone

For many years it has been known that thyroid hormone, as produced in the thyroid gland, has 4 iodine atoms attached to the molecule. It has also been known that a molecule containing only 3 iodine atoms is present in the circulation. The question is, is the T_4 a "prohormone" (storage or precursor) to the T_3, and is T_3 then the metabolically active form of the thyroid hormone? Dr. Jack Oppenheimer has attempted to answer these questions. He has shown that a drug, propylthiouracil, prevents the conversion of injected, radioactively labeled T_4 to T_3. Injected T_3 will be metabolized normally, however, in spite of the presence of the drug. This evidence is taken to mean that T_4 is actually the "prohormone" and that T_3 is the active material in the body. Studies in humans tend to confirm this hypothesis.

In order to determine the site of action of T_3 in the cell, small amounts of radioactively labeled T_3 were injected. Analysis showed that nuclear membranes contained labeled material, indicating that the nucleus is one of the earliest sites of activity of thyroid hormone. Since pituitary cells also show reaction to T_3, it is proposed that the pituitary activity for TSH production is controlled by the concentration of circulating T_3.

Pituitary Hormones and Hypothalamic Factors

In recent years it has become clear that factors produced in the hypothalamus of the brain (presumably by nervous stimuli) are responsible for control of production and release of the hormones from the pituitary. Much work is currently being done on the so-called releasing factors and inhibiting factors. This work includes studies to purify, characterize and synthesize the hypothalamic factors so that they may be used in clinical studies and in therapy.

Thyrotropin releasing factor (TRH) has been chemically characterized and synthesized. It is now being used in clinical studies by Dr. Robert Utiger (University of Pennsylvania) and others. Utiger has found that TRH increases the production of thyroid stimulating hormone (TSH), which in turn increases the amount of circulating T_3 and T_4. In addition TRH results in elevated serum prolactin concentrations, and is a more potent prolactin releasing hormone than it is a thyrotropin releasing hormone. This work was done in collaboration with Drs. Jacobs and Daughaday at Washington University in St. Louis.

Growth hormone (G.H.) is of particular interest to this Institute since we support the National Pituitary Agency which has as its main purpose the extraction of growth hormone from human pituitaries for clinical research studies in dwarfed children. That there is often interaction between various of the hormones is shown by the observation that injection of growth hormone sometimes causes a decrease in serum T_4 levels. Dr. Utiger has now found that G.H. administration results in an impairment of TRH stimulated TSH release, which in turn causes the above noted decrease in serum T_4 levels. The mechanism for the G.H. caused decrease in TRH is at present unknown.

Dr. Armen Tashjian has established cell cultures from pituitary glands and has isolated strains which produce either growth hormone or prolactin, but not both hormones. He notes that the G.H. producing cells respond to TRH (but not to gonadotropin releasing factors), and when thus stimulated increase prolactin production. The mechanisms involved are unknown at this time. In spite of the evidence presented above that TRH is a potent prolactin releasing factor, there is also evidence that there is prolactin releasing factor (PRH) distinct from TSH. This concept has been reinforced by Reichlin, who has shown the separateness of TRH and PRH by bioassay methods. He has to date been unable to elucidate the nature of the PRH. Arimura has also been working on PRH, with a different bioassay system. In addition, he is also working to characterize a prolactin inhibiting factor. This factor appears to prevent the release of prolactin from the pituitary, by an as yet undetermined mechanism.

In last year's report, we noted the purification and synthesis of luteinizing hormone releasing factor (LR-RH). Arimura has been studying this purified releasing hormone, and notes that it also causes release of follicle stimulating hormone (FSH) both *in vivo* and *in vitro*. So far, they have been unable to separate LH-RH and FSH-RH activity in the hormone. There is still a possibility of a separate FSH-RH, however, so the search for another releasing factor goes on.

In addition, studies are still being pursued in several laboratories for growth hormone and cortico-tropin releasing factors. There is evidence for existence of these factors, but they have yet to be purified.

Dr. Roderick Walter reports that a breakdown product of the posterior pituitary hormone oxytocin may be the releasing factor for melanocyte stimulating hormone (MSH). The releasing factor appears to be a peptide chain of 5 amino acids. The melanocyte stimulating hormone acts on pigment cells called melanocytes. T. T. Tchen of Wayne State University has found that a chemical, cytochalasin B, causes the formation of binucleated melanocytes in MSH-treated cells from goldfish. The two nucleii thus formed are morphologically different. These results show that MSH induced melanocyte formation involves differential mitosis. Furthermore, MSH and cyclic AMP (a second messenger) serve dual roles in the development of melanocytes. MSH seems to cause differential mitosis of the parent or stem cell and cAMP causes structural differentiation within the melanocyte. Tchen believes that these studies on melanocyte development are the first demonstration in higher animals that a hormone, acting via the second messenger cAMP, induces differential mitosis of a stem cell.

The importance of basic research to human medicine has been pointed out many times. What may seem of interest only to the "pure" scientist often leads to new treatment of patients. Dr. Joseph Meites reports that his studies on mammary and pituitary tumors in rats has led to current clinical trials with several central acting drugs in humans with breast and pituitary cancers. One drug in particular, l-dopa, shows promising results in inducing remission of breast cancer, and in decreasing prolactin release in patients with pituitary adenomas.

07 - METABOLISM PROGRAM AREA

One must look back to the significant biomedical developments of the last fifteen years to appreciate the role the metabolism program must play in the understanding and management of the important diseases of the day. Many of the breakthroughs in medicine have had their genesis in the program since it supports so much of the basic research necessary to understanding disease. Much of the work on cyclic AMP and the mechanism of its action in turning on and off metabolic activities as appropriate to the work of the cell is supported in the program. Affinity chromatography with its vast potential for isolation of biologicals is being exploited with program support.

Much attention is now directed to how the activities induced by cyclic AMP are reversed. Insulin plays an important role in this process but its direct action is confined to the exterior of the cell membrane. Thus it must exert its effects through second and third messengers and they may be many. One effect of insulin is to increase the permeability of cells to electrolytes, amino acids, and glucose. The polyamines, spermine and spermidine, are also concentrated by adipocytes under the influence of insulin. Thus, these nutrients may themselves have second and third messenger activity for insulin. This function is best illustrated by glucose, which can bind to cyclic AMP induced phosphorylase, changing its conformation in such a manner that it is rapidly hydrolyzed by a specific phosphatase. Phosphorylated glycogen synthetase is relatively inactive but it is readily hydrolyzed by a phosphatase to make it active once the hydrolysis of the phosphorylase is relatively complete. Evidence is also accumulating for active magnesium transport into the cell under the influence of insulin. Magnesium, calcium, manganese and spermidine have some preferential binding sites and others where the cations compete. One effect of insulin is to stimulate protein synthesis and this may depend on maintaining a delicate balance among the various cations.

The polyamines, spermine and spermidine, are being recognized increasingly for their role in cell growth and differentiation. Putrescine or diaminobutane is derived from ornithine by decarboxylation. The aminopropyl side chains are derived only from the essential amino acid, methionine, through decarboxylation of S-adenosylmethionine and transalkylation reactions entirely comparable to the trans-methylation reactions. The transalkylation reactions modify proteins, nucleic acids, and phospholipids profoundly in their stability and metabolic reactivity so limiting the supply of methionine or of polyamines or both to the cell is one way of controlling its metabolism and growth.

Reports in the literature are contradictory as regards the effects of extracellular polyamines. In some cases they appear as deadly poisons, in others as anabolic factors. It is rapidly becoming apparent that the effects of the polyamines are almost diametrically opposed to those of their oxidation products and that the diamine oxidase responsible for the oxidation is itself an important regulatory enzyme. This enzyme is a copper protein reacting directly with oxygen to give hydrogen peroxide and the aldehyde corresponding to the primary amino groups of the polyamine. Having two or more positive charges and one or two aldehyde functions, these aminoaldehyde oxidation products are thought to be the toxins in such diseases as tuberculosis, leprosy, and now cystic fibrosis. These products appear in the blood in cancer and appear to correlate with the degree of necrosis. They may also account for the cachexia of cancer. Their level in blood should be the resultant of such factors as the level of diamine oxidase, the rate of passage of polyamines into the blood, the clearance of the polyamines and their metabolites in the urine, etc. One could also expect variation in the concentration of carriers such as heparin.

The basic defect in cystic fibrosis is now suggested at the level of the intracellular diamine oxidase since intracellular levels of the polyamines are very high. Unfortunately, the study of the diamine oxidases has received much less attention than it deserves and the field is just now emerging from relative chaos. Optimism arises from the introduction of a new reagent, fluorescamine, which shows no ammonia blank and can quantitate primary amines readily in the nanamole range. Thus, future studies will use the natural substrate, the polyamines, rather than benzylamine which was more readily quantitated. At present, the nature of the cofactors for the cellular diamine oxidase is unknown. The plasma enzyme utilizes pyridoxalphosphate which can also transaminate with amines to give aldehydes or ketones and pyridoxamine. Presumably, the oxidase is merely reoxidizing the pyridoxamine to pyridoxalphosphate.

Benzylamine oxidase (presumably correlating with diamine oxidase activity) activity in cystic fibrosis fibroblasts is nearly ten times the normal level in the face of an approximate fourfold increase in polyamines. In some manner the oxidation of the polyamines is being blocked, and they are spilling into the circulation along with cell secretory products. A reasonable possibility is that the intracellular enzyme is similar in properties to proline oxidase, lysine oxidase, and the various hyroxylases of the endoplasmic reticulum which have such cofactors as pteridines, ketoglutaric acid, ascorbic acid, vitamin K, dopaquinone, etc. Characteristic of these factors is an ability to transaminate, converting primary amines to aldehydes or ketones. The aldehydes derived by lysine oxidase play a fundamental role in the cross linking of collagen and a similar role of polyamine aldehydes in setting the conformation of proteins is a reasonable expectation. It has already been demonstrated that vitamin K is necessary to setting the conformation of prothrombin and Stuart factor in a particular calcium binding mode with consequent regulation of the active site of the factors.

The activity of clotting factors can be determined in the laboratory and has permitted their partial purification and characterization. Additional factors such as somatomedin, third factor, diabetogenic factors, etc. have provided greater challenge. It is worth considering that their lability results from dissociation of a small prosthetic group such as a polyamine or aminoaldehyde.

The major impediment to understnading the role of polyamine oxidation products in metabolic regulation has been the lack of a suitable tracer. Most studies have been carried out with the tag in the putrescine moiety of the polyamine and give no indication of the number of times it has cycled from putrescine to spermidine to spermine through the transalkylation reaction and back to spermidine and finally putrescine through the diamine oxidase reaction and the cleavage to acrolein (vinylformaldehyde). Acrolein is highly reactive and polymerizes to unrecognizable condensation products with tyrosine, tryptophan, etc. This may be part of the insoluble "melanin" found in protein hydrolysates which is filtered off and dumped down the drain. Fortunately, C13 is becoming increasingly abundant and provides an ideal tracer which can be followed in the mass spectrometer or by nuclear magnetic resonance. Not until methionine tagged in the aminopropyl positions with C13 is available can one expect to have a real understanding of the cellular diamine oxidase and the intermediary metabolism of the polyamines.

In the meantime, much remains to be done with the fluorescamine method to assess the relation of plasma polyamine and diamine oxidase levels in various diseases. Ruminants have high enzyme levels and presumably correspondingly low polyamine levels. Heparin, pregnancy, and medullary carcinoma of the thyroid effect the release of connective tissue enzyme into the plasma. The polyamines and their oxidation products are emerging as fundamental parameters to be considered in such diverse conditions as insulin resistance, obesity, and the relation of diet to susceptibility to infection.

12 - KIDNEY DISEASE AND UROLOGY PROGRAM AREA

Introduction

Ten years have now passed since the National Institutes of Health decided to delineate in its extramural programs a category encompassing research and research training in the kidney and urological field, by assigning the responsibility to the NIAMDD for developing and maintaining this particular emphasis. At that time approximately 130 research grants and ten training programs were actively funded in the area of kidney and urologic disease. The Kidney Disease and Urology Program during Fiscal Year 1973 has better than 200 research grants and 41 training programs actively funded. Without minimizing the importance of the lower urinary tract it should be noted that about 75 percent of the research grants funded through Program 12 deal with basic physiological investigations of the kidney. However, increasing emphasis and interest is becoming apparent by the slow but gradual increase of requests for support of investigations dealing with clinical problems such as chronic renal disease, uremia, urolithiasis, pyelonephritis, glomerulonephritis, diabetes insipidus, and polycystic disease.

Kidney Transplantation

The results with clinical renal transplantation today are reasonably good with a seventy percent five-year survival rate using related donors and fifty percent two-year survival utilizing cadaveric donors; however, these survival rates are not any longer than they were four or five years ago. What advances have been made have related to organizing regional programs for cadaveric retrieval so that *more* people can be transplanted. The major cause of transplant failure is the current immunosuppression which is *not* selective and renders the patient immunologically incompetent, a prey to opportunistic infections. Recent findings by a Stanford group indicate that survival of rat kidney transplants can be lengthened by an "enhancing antibody," prepared from splenic mononuclear cells. These findings now make possible for the first time the preparation of a reagent capable of enhancing specific immunologic unresponsiveness.

Another group at the University of California Medical Center, San Francisco, has been selected to study a highly potent rabbit anti-human thymocyte globulin in a double blind prospective trial in patients receiving cadaver kidneys. Excellent evidence has already accumulated which indicates that this substance is highly effective in significantly prolonging skin graft survival in primates. In addition, another NIAMDD grantee at another institution has demonstrated clinically that the anti-human thymocyte globulin results in an 80 percent cadaver graft survival at one year, which is an appreciable increase over the current 50 percent survival rate utilizing standard immunosuppressive techniques.

Basic Physiological Investigations

Areas of basic kidney investigation, which seem to have gained new impetus, are the tubular chloride transport mechanism and the plasma membrane adenylate cyclase-cyclic AMP system.

For many years, the renal community accepted the idea that chloride movement was passive and was secondary to active sodium transport. However, two exciting new reports of possible active chloride transport from the thick ascending limb of the Loop of Henle were recently reported at the Vth International Nephrology Congress. In both reports, one given by an NIAMDD grantee, net sodium chloride transport from the lumen of the ascending limb was demonstrated and mean transmembrane potential difference on the lumen side was positive. If these preliminary studies are confirmed, it would mean that solute transport out of the ascending limb is substantially due to active chloride transport. However, understanding of the processes underlying proximal tubular chloride transport remains difficult, especially because of the continuing doubts about the existence of a negative trans-tubular potential difference and the fact that a very small negative potential would suffice to drive chloride across the proximal tubular wall because of its low electrical resistence.

The kidney adenylate cyclase-cyclic AMP system has been implicated in the mediation of parathyroid hormone, vasopressin and catecholamine action. Studies indicate that distribution of target cells and specific receptors for the various hormones exist in different regions of the kidney. Therefore, it has been proposed that hormones act through the second messenger, cyclic AMP, by regulating kidney cell activity via endogenous plasma membrane subunits, such as adenylate cyclase and cyclic AMP-dependent protein kinase. This view is of considerable interest since kidney epithelial cells are highly differentiated for active and passive membrane transport activities. Furthermore, the involvement of specific plasma membrane components in endocrine regulation of processes associated with the surface membrane is thought to be rational since vasopressin and parathyroid are involved in regulating discrete membrane parameters such as permeability and ion translocation.

The support from this Institute for studies by one investigator is helping to clarify the nature and management of diseases that offset fluid balance. For instance, he has produced a detailed analysis of various inherited causes of ADH-resistant urinary concentrating defects in mice. By combining the knowledge of the solute composition of renal tissues and urine, with the geometric analysis of the renal countercurrent system he envisions the possibility of identifying different causes which, singly or in combination, lead to defective urinary concentration in several genotypes of mice. The work of this project appears to have contributed to the biomedical field in two major ways. The first, by the utilization of inherited diseases in animals, as experimental models to answer fundamental physiological questions and as analogue models of human disease. The second major contribution of this project has centered about the use of mutants and of hybrids to define causes of both normal function and of disease.

International Congress

Support of investigators for travel to the Vth International Congress of Nephrology, which was held in Mexico City during the week of October 8-14, 1972, was in part provided by both the NIAMDD and the NHLI. The American Society of Nephrology had foregone its regular annual meeting and consequently both the quality and number of scientific papers presented can in large measure be attributed to the huge American delegation. In addition, there were several well-presented symposia covering topics which are currently being extensively investigated, including renin secretion, natriuretic hormone(s), dialysis, renal hypertrophy, nephropathies of systemic diseases and calcium, phosphorus, and vitamin D in relation to uremia. The site of the congress was at the National Medical Center of the Mexico Social Security Institute, which easily accommodated the 2400 registrants and allowed the interpersonal exchange of information and ideas.

Training

The number of training grants supported by this program rose to its highest level of 41 during Fiscal Year 1973, including two hardship extensions to permit trainees to complete their period of training. Five new programs were initiated during this period. Of the 41 training awards 15 were in Urology, 21 were made to Medicine Departments, 3 to Pediatrics Departments and 2 to Physiology Departments.

During the same period there were six active Research Career Development Awards, seven Postdoctoral Fellowship Awards and eighteen Special Fellowship Awards.

In keeping with the directive to phase out the training and fellowship programs, the spring meeting of the Renal Disease and Urology Training Grants Committee was cancelled.

06 - HEMATOLOGY PROGRAM AREA

Introduction

Research in hematology supported by the extramural grant program in this Institute can be characterized by defining the activities in relation to one or more of the following areas:

(1) the non-genetic anemias (e.g., nutritional deficiency anemias, acquired hemolytic anemia) and disorders of blood cell production (e.g., aplastic anemia, anemia of chronic renal failure)

(2) The genetic anemias including genetic disorders of hemoglobin and its production (e.g., sickle cell anemia, thalassemia)

(3) the enzymatic and metabolic defects of all blood cells (e.g., red cell glucose-6-phosphate dehydrogenase deficiency, chronic granulomatous disease)

(4) the immunology of bone marrow transplantation and of autoimmune hematologic disease

A recent analysis of the frequency of hematologic disorders seen by clinical hematologists reveals that about 40% of patients are suffering from some sort of anemia. In addition, the course and prognosis for other diseases can be adversely influenced by attendant hematologic problems such as the chronic anemia secondary to renal insufficiency, malignancy and gastrointestinal and endocrine disorders. The magnitude of the problems of anemia is well recognized by the hematology program in which close to 50% of the total number of projects supported deal with red cell production and the anemias.

Non-Genetic Anemias and Disorders of Blood Cell Production

Studies of iron metabolism continue to receive emphasis as reports accumulate documenting iron deficiency anemia and as controversy continues over the proposed but still unadopted Food and Drug Administration regulation for the increased level of fortification of bread with iron. Timely work in this area concerns the screening of potentially useful oral iron supplements. This investigation has recently shown the superior absorption of the ferric fructose complex compared to ferrous sulfate and suggests that polynuclear iron might be generally more effective as an iron supplement than ferrous or ferric salts or small molecular iron (III) chelates. Bread fortified with ferric fructose or ferric citrate at 38 mg Fe/kg produced a steady, significant increase in liver ferritin iron stores in mice.

Progress has been made in the past year in the differential diagnosis of the megaloblastic anemias with the development of a radioassay for serum folate. Folic acid deficiency is seen most commonly as a result of malnutrition and associated with such disorders as malabsorption syndromes, alcoholism, pregnancy, during the course of chronic hemolytic anemias and secondary to drugs, particularly Dilantin and oral contraceptives. The specific and sensitive assay developed will contribute to clinical evaluation of disorders of folate metabolism.

Acquired hemolytic anemia is characterized by an increased rate of destruction of the red cells by interaction with an environmental influence. Small molecules from various sources such as medicinal agents, agricultural chemicals and pollutants of the environment are being intensively studied by an NIAMDD grantee for their propensity to cause hemolytic anemia and ferrihemoglobinemia. Knowledge of their mode of action will be useful for prevention and therapy of these obscure but potentially lethal anemias.

A previously reported test for red cell autoantibodies in autoimmune hemolytic anemia (the complement-fixing antibody consumption test) has been modified to permit application of the test to infants. This "mini"-system was achieved by a 5-fold reduction in scale requiring only 0.2 ml of packed red cells. This test has facilitated diagnosis in those patients in whom the standard antiglobulin test (Coomb's test) gives a negative reading.

In vitro studies of bone marrow and blood cell growth in semi-solid media are providing new evidence about such disorders of production as myelofibrosis. Because of the abnormal proliferation of blood cell precursors in the marrow, the disease closely resembles chronic leukemia and ways have been sought to differentiate them in order that suitable therapy can be initiated. Patients with myelofibrosis were recently found to have a marked increase in the number of circulating stem cells, a clearly different finding from that in patients with chronic myelogenous leukemia (CML). Colonies grown in semi-solid media were not only more numerous than in CML but were also larger. This culture system, developed only recently, also facilitates study of colony stimulating factor (CSF), a humoral or hormonal substance affecting white cell production in a manner that may be analogous to how erythropoietin governs red cell production. Much remains to be learned about the relationship of CSF to the control of granulopoiesis in normal individuals before the culture system can be exploited to develop better treatment for disordered white cell production.

Genetic Disorders of Hemoglobin

Eight projects are presently being funded from the sickle cell disease earmark of NHLI. In addition 17 grants with primary emphasis on studying this disorder are being supported by NIAMDD funds. The intensive attack on this severe chronic hemolytic disease includes

(1) research relating differences in the primary structure of the hemoglobin molecule to function,

(2) studies investigating the mechanisms of red cell membrane injury that lead to the formation of irreversibly sickled cells and the metabolic consequences to the cell of the sickling-desickling process,

(3) projects working on therapeutic aspects of preventing the disease or ameliorating the hemolytic crises.

Progress in gathering structural information is being made by an investigator using the cysteine residues of the hemoglobin molecule as sites for probes (spin labels) which are sensitive to changes in protein conformation. This should permit monitoring the transition of deoxyhemoglobin S as it goes from the soluble to insoluble state thereby providing information on the dynamics of the sickling process and how it may be controlled.

Looking at a different part of the sickled red cell, another investigator has found an accumulation of calcium in the membrane of oxygenated hemoglobin S cells undergoing metabolic depletion with a corresponding decrease in membrane elastic properties. The results suggest that loss of elasticity of these membranes "fixes" the cells in a permanently sickled configuration due to the formation of bridges between the adjacent protein layers of the internal aspect of the red cell membrane.

New approaches to therapy are receiving intensive study. One group of investigators is following up on its original observation that cyanate is effective in inhibiting in vitro red cell sickling by studying the mechanism whereby this occurs. Work in the past year indicates that the site of action is the NH_2-terminal valine residues of hemoglobin S. Another group has found that nitrogen mustard inhibits gelling and sickling of hemoglobin S in vitro. Still another approach to therapy is under study following the observation that patients with both the red cell enzyme deficiency G6PD and sickle cell disease have a 3-fold increase in survival over those with sickle cell disease alone. The mechanism underlying this apparent protective effect is being investigated.

Drs. Helen Ranney and Harvey Itano, NIAMDD grantees, were honored during the past year with the Dr. Martin Luther King award for their contributions to research on sickle cell anemia.

Following the passage of the Cooley's Anemia Act, Public Law 92-414, initial steps have been taken to begin implementation of a concerted program. An Inter-Institute Committee has been formed to draw together the efforts already going on in the area supported by various funding units at NIH. Besides the National Institute of Arthritis, Metabolism, and Digestive Diseases, the National Heart and Lung Institute, the National Institute of Child Health and Human Development, the National Institute of General Medical Sciences, and the Division of Research Resources are represented on the Committee which also maintains contact with the efforts in the Health Services and Mental Health Administration on the screening and counseling aspects of the legislation.

High priority areas of research dealing with thalassemia were identified by scientists working in the field at an advisory meeting and at a Cooley's Anemia Workshop sponsored by NIAMDD and held in conjunction with the 3rd Cooley's Anemia Symposium in New York City. Possible approaches to therapy through correction of the α/β -globin chain imbalance, blocking or reversing the fetal to adult hemoglobin switch or through actual incorporation of the β-globin gene into the cells were discussed. Screening and counseling considerations were also carefully explored.

Research grant support identified as working towards understanding the molecular basis of thalassemia and developing appropriate therapy was awarded for 13 projects. Two of these were newly supported efforts. Two foreign projects are supported, one in Thailand and one in Italy, regions where the incidence of thalassemia is particularly high. The population group being studied in Ferrara, Italy, is particularly unique since affected individuals there synthesize no β chain hemoglobin at all in contrast to most Cooley's Anemia victims elsewhere. Comparison of these two types of thalassemia may yield significant data on the nature of the defect.

Enzymatic and Metabolic Cellular Defects

Red cell survival studies have been done which demonstrate that in roughly one-half of uremic patients a plasma factor accumulates which inhibits red cell hexosemonophosphate shunt (HMPS) metabolism and thereby shortens red cell survival times. Furthermore, the red cell defect was potentiated by contaminants in tap water hemodialysis baths and hemolysis worsened in patients so dialyzed. As in patients with red cell G6PD deficiency, another enzyme of the HMPS, explosive Heinz body hemolytic anemia was found to occur in affected uremic patients taking oxidant drugs such as the sulfonamides and antimalarials. These studies suggest that uremic patients be screened for red cell HMPS deficiency prior to administration of oxidant drugs and when found to be affected dialyzed only against purified water baths.

Investigation of granulocytes from jaundiced cirrhotic patients showed a basal rate of glucose metabolism significantly increased over that of normal granulocytes or of granulocytes from these same patients studied in normal plasma. When the same cells had their rate of glucose metabolism measured during phagocytosis however, differences could not be detected. This indication that cirrhotics have a plasma factor that affects the basal rate of glucose metabolism will be pursued by studying the effects of bilirubin on glucose metabolism.

Important new insights into the role of the red cell membrane in cellular metabolism were made by a grantee studying the effects of vitamin E deficiency on the red cell's susceptibility to peroxidation. In addition to describing the mechanism leading to the rapid changes in membrane composition and characteristics that could be related to the membrane permeability abnormalities seen in hereditary hemolytic anemia, a test for the differential diagnosis of obstructive jaundice in the newborn has been developed. The clinical utility of this test, the erythrocyte hydrogen peroxide hemalysis test (PHT), is based on the premise that congenital biliary obstruction with absent bile salt excretion must lead to red cell membranes deficient in vitamin E. When red cells deficient in vitamin E are incubated with hydrogen peroxide, hemolysis occurs.

Immunology of Bone Marrow Transplantation

Despite strides in the technology of human lymphocyte antigen and mixed lymphocyte culture antigen matching for histocompatibility, grant-versus-host disease remains a grave problem in bone marrow transplantation efforts. The relatively few projects in bone marrow transplantation being supported by this program deal with mouse and dog models. Replacement therapy of hemopoietic cell transplantation is of potential use in sickle cell anemia, aplastic anemia, acquired agranulocytosis, inherited and acquired deficiencies of the immune system and possible leukemia. In the past year, supported studies have indicated that in mice the host parameters of the allograft reaction are different for hemopoietic and skin grafts. This will undoubtedly affect the mechanism by which potential donors are selected for transplantation of marrow.

Because of reported success in achieving organ transplantation through the use of anti-lymphocyte globulin (ALG) to mediate the rejection process, another investigator is studying ALG effects on stem cell renewal in irradiated mice. Hemopoietic colonies produced by marrow pretreated with ALG were fewer in number and tended to be smaller than those produced by untreated marrows. These preliminary studies seem to indicate that immunosuppressive antisera may have an adverse effect on hemopoietic tissue and be deleterious to transplanted marrow.

Training

The number of training grants supported in this program fell to 32 in fiscal year 1972 including one hardship extension to permit trainees on board to complete their period of training. One new program was funded this year. One hundred and eleven postdoctoral trainees and fourteen off-quarter students received training in hematology.

The total number of Research Career Development Awards increased over last year's level by one although there were eight new starts during this period.

The winter and spring meetings of the Heamtology Training Grants Committee were not held as the phasing out of training programs resulted in applications not being considered for review.

OFFICE OF THE ASSOCIATE DIRECTOR FOR EXTRAMURAL PROGRAM ACTIVITIES

ASSOCIATE DIRECTOR'S REPORT

This Office has been created in the reorganization of the Institute's Extramural Programs occasioned by Public Law 92-305 changing the name of the Institute and establishing certain new positions and Committees in the Institute.

Implementation of the changes desired has been slow and conceptual rather than established in administrative and personnel language up to the present time. Procrastination to effect these changes has been compounded by failure to receive an appropriation containing additional positions, early authorization to implement this organization, plus general personnel restrictions regarding appointments to fill vacancies, and slowing of promotions to prevent "grade creep."

Eventually it is hoped that this office will have an Administrative Officer directly responsible to the Associate Director, a Grants Management Officer essentially as at present, a data analysis, storage and retrieval "officer" and an "operations" officer. The first of these has been functional since last October but is nominally still related directly to the Grants Management operation, an unsatisfactory relationship for the long run. The Grants Management Officer's post is to be redescribed in line with the reorganization. We have been without the services of an equivalent to the "data analysis, storage and retrieval" officer for over three years now, with the Associate Director continuing to give less than satisfying service in this post as "acting." The job as operations officer will require some changes in job description as a consequence of reorganization and more lately the phase-out of training and fellowship support programs.

The work of this office continues to be supportive in nature for all the programs of Extramural Activities. Thus, we provide personnel services to the Extramural Programs as middlemen between the Institute Personnel Office and our operating levels; administrative services re space allocation, procurement of services and supplies in a similar fashion through the Institute Administrative Office, grant and award processing for all "clusters" and program areas, and the like.

Under the circumstances prevailing we feel fortunate to have more or less satisfactorily accomplished our ends, frequently not on the time schedule we hoped for but eventually, now and then less completely and effectively than we wished. It must be evident that morale, hope and promises--none of these are as high as we want them to be but we take some solace in that none has failed ...yet.

ANNUAL REPORT OF TRAINING AND FELLOWSHIPS OFFICER - NIAMDD-EP

Reflecting the contrasting activities before and after release of the FY 74 budget news, this report summarizes the year's two main planning efforts: one developed in preparation for November Council as a means of more effective training fund allocation, the other designed to keep training concerns in view even without training funds.

The November plan was the result of deliberation involving representatives of the Institute training review committees and Advisory Council. It seems worthy of record even though never implemented. The attendant discussion was noteworthy for the recognition accorded to new sources of initiative in determining program priorities and for the willingness displayed to tackle old stumbling blocks in accommodating individual grant budgets to the broad fiscal picture. Willingness probably owed much to recognition. Starting from general discussion of the institute's reorganization with special emphasis given to digestive diseases and nutrition, the advantage accruing to these programs emerged as of two sorts: additional funds inevitably limited to the special emphasis group and a specified allocation, a planning element that might be approximated for all. In place of the customary direct step from November Council review of the year's competing business to an Institute funding plan, representatives from the review committees proposed an intermediary step, one that would allow them a second look at individual grant budgets with opportunity to propose revision in the light of probable program allocation. To the extent that this interest of the initial review group could be developed so as to evoke from both levels of review explicit statement of underlying principles governing allocation decisions, it seemed well worth trying. The plan adopted included the following elements:

(a) Preparation for November Council of a summary by program of competing business approved for July 73 in terms of

- number of training positions;
- stipendiary costs;
- first estimate of non-stipendiary investment.

(b) Review of this material with Council in the light of background material that would include

- comparable data on previously established commitments;
- information on current utilization of the established positions;
- information on fellowship support, grouped to distinguish those fellowships complementing and extending the training grant-supported effort from those providing support with no parallel in the training grant-supported centers;
- a summary of findings gleaned from exit documents submitted in the past 12 months by both trainees and fellows;
- the customary budget data.

(c) On this basis staff with Council advice would arrive at recommendations for each program in terms of

- number of positions to be established from the approved competing applications and from this the stipendiary investment;
- non-stipendiary investment, worked out as a compromise between customary initial review group assessment and estimates of funds available;
- number of centers to be supported - specified perhaps as a range so as to allow the initial review group members some leeway in their "second look."

The training committees would have their "second look" at their Spring meeting, utilizing the Council determinations together with any more recent fiscal information. Their contribution based on this material would depend for its effectiveness on success in bringing out criteria and principles. Focal points envisioned include the following:

- how to adapt the budgeting principles to be applied to applications examined individually to the budgetary limitations governing the program as a whole;
- how to reconcile self-estimates of a number of training positions for a particular field with limitations imposed at the supply end by inelastic manpower pools and with opportunity at the other end as ascertained through review of exit documents and surveys such as the recent hematology study undertaken by Dr. Helen Ranney.

With its apparatus of applications, budgets, committees, the foregoing account is certainly "dated," and, in a phase of indefinite duration marked by lack of that apparatus, even superfluous. To the extent that the continuing opportunity for investigator development depends upon active cooperation between research community and supporting agency, the nature of that effort as of, say 1974, may be expected to show significant elements of continuity with our position in 1972, changes in support mechanisms notwithstanding. The procedural changes embodied in the November plan reflect a growing willingness, once the effective research training centers have been identified, to refer decisions as to numbers supported (of both centers and trainees) and dollars invested to a broader context than that available from individual grant review.

To develop that context as a basis for the decisions ahead we need to learn something of the institutional adjustments to the phaseout now underway. This we propose to accomplish by bringing some segment of the continuing training endeavor whatever its support into clearer view. Specifically, we plan a sample-based study of postdoctoral training in a small group of institutions. The sample will include some heavy users of our training support instruments, some light, the nuclear group comprising those institutions who either have utilized or expressed strong interest in utilizing our multicategorical support. (The key consideration here was not some special virtue attaching to multicategorical support, but rather the attendant interest in exploring with us the problems underlying usual training support.)

The study would focus on individuals in graduate training in clinical divisions (straight clinical as well as research) starting with enumeration of those spending a year or more in association with such divisions for training purposes, and extending to consideration of support of their training (stipendiary and other) and its content, examining interaction between these. Data of this sort for, say 1972, 73 and 74, is expected to provide a context for examining the contribution of existing NIH support, for developing principles that could guide the design and introduction of auxiliary support mechanisms.

Administrative Services Section

In addition to the usual administrative services, this section also is responsible for the details involved in the travel for both the staff and consultants. Although the phase-out of training and fellowship grants will be accompanied by a corresponding decrease in related travel, it is anticipated that a part of this decrease will be offset by staff travel to site visits, meetings, conferences, etc. which the staff has been unable to attend previously. Consequently, the travel work load of this office is not expected to decrease by any substantial amount.

The space management function of this section has been demonstrated in FY 1973 with the re-arranging of office spaces to more closely relate to the "cluster" concept of the Extramural Programs new organization. With this has been the designation of an area for ultimate use as a conference room and the subsequent furnishing of this space as well as the office of the Associate Director for Extramural Program Activities. It is expected this work will be completed by early FY 1974.

A much-needed re-staffing of the Administrative Services Section was completed in FY 1973 with the employment of an Administrative Officer and additional clerical support. It is anticipated that those functions of this Section, which due to lack of personnel have been left undone, will now be given the necessary appropriate attention.

ANNUAL REPORT OF GRANTS MANAGEMENT BRANCH - NIAMDD-EP

The most apparent change in GMB this past year was the transfer of the Award Unit from the Operations Branch to GMB. This transfer was part of the overall Extramural Programs reorganization. The transfer included four people as well as their functions in the award process. Their particular functions include the initial steps for securing computer prepared award notices as well as the preparation of Approval (Encumbrance) Lists for all types of awards. It was felt that since this function directly followed GMB's input, and since it was the final step in the award process, that it legitimately belonged within GMB.

Again this year we were faced with having to cope with the problem of not knowing our appropriation level. This was compounded by the fact that we were at first assured of having at least the President's budget level for research grants of $97,359,000. Later we were informed that our operating level would be the FY 72 level of $93,616,000. Subsequently, we were informed that we were reduced to $91,312,000. This information was not available until after our June and November Council. Consequently the pay plans established were based on the higher appropriation level. Since the effect of this was to leave almost no funds with which to pay March Council approvals, we petitioned OD-NIH for permission to reduce our commitments by an average of 10%. This permission was granted with the understanding that commitments for the entire remaining project periods would be reduced; not just FY 73 commitments. With this permission we were able to assign "A" pay codes to a fairly respectable number of March Council approvals. Ultimately it is expected total research grants funded will approximate 28-30 percent of the number recommended for approval.

Another major decision was to phase out Training grants and Fellowships awards. This decision included major reductions in our Training and Fellowship funds. This has forced GMB into much closer scrutiny of these types of awards to assure that phase out guidelines are being followed. In particular, this involves large reductions of committed levels in order to stay within the funds available and the shortening of committed periods of support to allow only up to 24 months of stipend support to committed trainees, except under most unusual circumstances.

In the realm of personnel, we are still experiencing some problems; although the addition of four people did help alleviate some clerical problems. Our biggest need continues to be the strengthening of our Grants Management Specialist staff along with securing additional help in the fiscal management area. Present personnel policies preclude the achievement of these desirable ends.

ANNUAL REPORT OF THE GRANTS OPERATIONS GROUP - NIAMDD-EP

The Grants Operations group has felt the impact of Extramural reorganization with the transfer of the Encumbering and Awarding Unit to the Grants Management Branch. The primary responsibilities of the group now are concerned with pre-Council activities, Council operations, and the mail and files handling.

There are only eight people in the group, but with the phase-out of the training programs, the work involved in processing grant applications and the sorting and collating of material for Council review will be at a more manageable level.

TABLE I

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Support of Research Grants

Program Number	Area Name	Actual July 1, 1971--June 30, 1972 No. Grants	Actual July 1, 1971--June 30, 1972 $ Encumbered	Estimated July 1, 1972--June 30, 1973 No. Grants	Estimated July 1, 1972--June 30, 1973 $ Encumbered
01	Arthritis	147	8,632,971	151	8,464,000
02	Dermatology	59	2,997,827	55	2,847,000
03	Diabetes	113	5,663,596	116	5,581,000
04	Endocrinology	231	11,795,694	225	11,986,000
05	Digestive Diseases	215	11,078,265	218	10,855,000
06	Hematology	181	8,595,923	185	8,697,000
07	Metabolism	403	19,658,783	336	17,211,000
08	Nutrition	137	6,187,931	125	6,260,000
09	Orthopedics	91	4,999,817	89	5,000,000
10	Physical Biology & Related Areas	81	3,666,320	82	3,534,000
12	Kidney Disease & Urology	200	10,315,735	203	10,897,000
	Totals	1,858	$93,592,862	1,785	$91,332,000

EPO - 5/29/73

TABLE II

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Support of Research Training Grants

Program Number	Area Name	Actual July 1, 1971--June 30, 1972 No. Grants	Actual July 1, 1971--June 30, 1972 $ Encumbered	Estimated July 1, 1972--June 30, 1973 No. Grants	Estimated July 1, 1972--June 30, 1973 $ Encumbered
01	Arthritis	27	1,447,270	26	1,203,000
02	Dermatology	23	1,491,968	22	1,256,000
03	Diabetes	12	568,196	10	429,000
04	Endocrinology	42	2,333,566	38	1,766,000
05	Digestive Diseases	31	2,102,646	32	1,760,000
06	Hematology	32	1,944,106	26	1,469,000
07	Metabolism	21	1,238,055	17	891,000
08	Nutrition	8	567,706	10	537,000
09	Orthopedics	18	636,934	16	469,000
10	Physical Biology & Related Areas	7	923,385	4	427,000
12	Kidney Disease & Urology	36	1,818,168	35	1,543,000
	Totals	257	$15,072,000	236	$11,750,000

EPO - 5/29/73

TABLE III

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Support of Postdoctoral Fellowships (F02)

Program Number	Area Name	Actual July 1, 1971--June 30, 1972 No. Grants	Actual July 1, 1971--June 30, 1972 $ Encumbered	Estimated July 1, 1972--June 30, 1973 No. Grants	Estimated July 1, 1972--June 30, 1973 $ Encumbered
01	Arthritis	4	31,500	4	31,500
02	Dermatology	3	27,500	1	7,000
03	Diabetes	12	82,608	5	37,500
04	Endocrinology	17	131,673	13	101,000
05	Digestive Diseases	8	62,363	8	69,000
06	Hematology	5	42,479	5	42,000
07	Metabolism	43	341,688	25	222,000
08	Nutrition	6	41,250	5	37,500
09	Orthopedics	3	22,000	3	23,500
10	Physical Biology & Related Areas	4	26,038	2	16,000
12	Kidney Disease & Urology	9	76,378	6	50,000
	Totals	114	$885,477	77	$637,000

EPO - 5/29/73

TABLE IV

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Support of Special Fellowships (F03)

Program Area		Actual July 1, 1971--June 30, 1972		Estimated July 1, 1972--June 30, 1973	
Number	Name	No. Grants	$ Encumbered	No. Grants	$ Encumbered
01	Arthritis	11	136,355	5	66,000
02	Dermatology	8	98,300	7	75,000
03	Diabetes	5	55,770	4	49,000
04	Endocrinology	20	238,223	10	129,000
05	Digestive Diseases	10	107,430	1	12,500
06	Hematology	8	115,982	3	44,000
07	Metabolism	8	95,721	7	93,000
08	Nutrition	3	35,392	2	27,000
09	Orthopedics	6	84,699	4	59,000
10	Physical Biology & Related Areas	1	9,600	1	11,000
12	Kidney Disease & Urology	20	217,059	10	120,500
	Totals	100	$1,194,531	54	$686,000

EPO - 5/29/73

TABLE V

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Support of Research Career Development Awards (K03 or K04)

Program Area		Actual July 1, 1971--June 30, 1972		Estimated July 1, 1972--June 30, 1973	
Number	Name	No. Grants	$ Encumbered	No. Grants	$ Encumbered
01	Arthritis	12	280,804	12	282,000
02	Dermatology	5	103,627	5	104,000
03	Diabetes	12	271,658	13	295,000
04	Endocrinology	13	345,156	14	312,000
05	Digestive Diseases	18	431,185	19	420,000
06	Hematology	15	370,650	14	328,000
07	Metabolism	18	387,927	18	382,000
08	Nutrition	5	108,993	3	58,000
09	Orthopedics	2	45,000	2	46,000
10	Physical Biology & Related Areas	5	120,150	2	35,000
12	Kidney Disease & Urology	4	97,000	4	95,000
	Totals	109	$2,562,150	106	$2,357,000

EPO - 6/4/73

TABLE VI

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Support of Research Career Awards (K06)

Program Number	Area Name	Actual July 1, 1971--June 30, 1972 No. Grants	Actual July 1, 1971--June 30, 1972 $ Encumbered	Estimated July 1, 1972--June 30, 1973 No. Grants	Estimated July 1, 1972--June 30, 1973 $ Encumbered
01	Arthritis	2	30,092	1	30,000
02	Dermatology	0	0	0	0
03	Diabetes	1	31,320	1	32,000
04	Endocrinology	12	364,204	11	333,000
05	Digestive Diseases	0	0	0	0
06	Hematology	1	31,050	1	32,000
07	Metabolism	5	155,567	5	161,000
08	Nutrition	3	86,801	3	91,000
09	Orthopedics	0	0	0	0
10	Physical Biology & Related Areas	0	0	0	0
12	Kidney Disease & Urology	0	0	0	0
	Totals	24	$699,034	22	$679,000

EPO - 6/4/73

TABLE VII

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Support of Academic Career Development Awards (K07)

Program Number	Area Name	Actual July 1, 1971--June 30, 1972 No. Grants	Actual July 1, 1971--June 30, 1972 $ Encumbered	Estimated July 1, 1972--June 30, 1973 No. Grants	Estimated July 1, 1972--June 30, 1973 $ Encumbered
01	Arthritis	0	0	0	0
02	Dermatology	0	0	0	0
03	Diabetes	0	0	0	0
04	Endocrinology	0	0	0	0
05	Digestive Diseases	5	133,920	5	142,000
06	Hematology	0	0	0	0
07	Metabolism	0	0	0	0
08	Nutrition	1	29,160	2	62,000
09	Orthopedics	0	0	0	0
10	Physical Biology & Related Areas	0	0	0	0
12	Kidney Disease & Urology	0	0	0	0
	Totals	6	$163,080	7	$204,000

EPO - 6/4/73

TABLE VIII

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Support of Clinical Investigator Awards (K08)

Program Area		Actual July 1, 1971--June 30, 1972		Estimated July 1, 1972--June 30, 1973	
Number	Name	No. Grants	$ Encumbered	No. Grants	$ Encumbered
01	Arthritis	0	0	0	0
02	Dermatology	0	0	0	0
03	Diabetes	0	0	0	0
04	Endocrinology	0	0	0	0
05	Digestive Diseases	5	132,829	8	207,000
06	Hematology	0	0	0	0
07	Metabolism	0	0	0	0
08	Nutrition	4	102,476	5	130,000
09	Orthopedics	0	0	0	0
10	Physical Biology & Related Areas	0	0	0	0
12	Kidney Disease & Urology	0	0	0	0
	Totals	9	$235,305	13	$337,000

EPO - 6/4/73

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS AND SCIENTIFIC COMMUNICATION

SCIENTIFIC COMMUNICATIONS SECTION

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in Renal Disease Workshop

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University Group Diabetes Program - Assessment of the
Clinical Trials of Oral Antidiabetic Agents

The Scientific Communications Section is charged with the general programming and planning of communication activities concerned with the various scientific areas under the purview of the NIAMDD. The present activities can be roughly divided into four main categories:

1. Current awareness literature services;
2. Retrospective literature studies;
3. Conferences and workshops; and
4. Other aspects of communication, such as the production of motion pictures, thesaurus development, etc.

Current awareness literature services are provided by the Institute in several specialized areas. There are four publications which provide subject organized listings of literature citations and one publication which expands to include abstracts. GASTROENTEROLOGY ABSTRACTS AND CITATIONS, now in its seventh volume, is intended to provide selected abstracts and literature citations in all aspects of gastroenterology, biochemical or clinical. Additionally, this year, complete coverage of four selected gastroenterological areas is being performed and compiled into formated magnetic tapes, suitable for searching through the facilities at DCRT. This additional information, along with the regular issues of Gastroenterology Abstracts and Citations, are being utilized by the Digestive Diseases and Nutrition Program of the NIAMDD.

The ARTIFICIAL KIDNEY BIBLIOGRAPHY (seventh volume), ENDOCRINOLOGY INDEX (fifth volume), DIABETES LITERATURE INDEX (eighth volume), and INDEX OF DERMATOLOGY (fifth volume) all contain citations to current world-wide literature which have been included by the National Library of Medicine in their Medical Literature Analysis and Retrieval System (MEDLARS). In order to serve those who are interested in highly specific areas, we have cooperated with the National Library of Medicine and four universities--Minnesota, Rochester, Case Western Reserve, and Vanderbilt--to produce structured, hierarchically-arranged indexes which function as in-depth current-awareness tools for easy review of particular subjects. The standardized terminology used in MEDLARS makes these publications invaluable for retrospective literature searching.

The Index of Dermatology was established and developed in cooperation with the National Library of Medicine over a period of three years by Universities Associated for Research and Education in Pathology under a grant from NLM. This grant expired in June, 1971, whereupon five dermatology societies jointly sponsored its publication until March, 1972. To further the NIAMDD Dermatology Program as well as respond to the increased interest and activity in dermatology, NIAMDD undertook the publishing of Index of Dermatology in April, 1972.

An Editorial Advisory Committee of fourteen outstanding scientists working in the area of dermatology has been assembled to ensure the continuation of the high quality of this publication.

Expansion of the Artificial Kidney Bibliography is underway to include other aspects of relevance to research on artificial kidneys such as kidney diseases and nephrology. An experimental edition was assembled and a meeting of the Editorial Advisory Committee was held to discuss the experimental edition. MEDLARS searches were designed to include the recommendations of the Editorial Advisory Committee. After format revision and inclusion of further suggestions by the Editorial Advisory Committee, the first experimental issue was printed. A meeting of the Committee will be held in June, 1973 to discuss this first experimental issue. Work with the International Committee for Nomenclature and Nosology of Renal Disease is progressing to include as much of the International Committee's nomenclature in the bibliography as feasible. Work with the National Library of Medicine's Medical Subject Headings Section is underway to include kidney-related vocabulary in the National Library of Medicine's computerized vocabulary. Clearance from the Office of Management and Budget to publish the new Kidney Disease and Nephrology Index as a recurring bibliography has been requested.

Conferences and workshops which are sponsored by NIAMDD are planned and organized by the Scientific Communications Section. In addition to the organization, which usually includes physical arrangements as well as program planning with the conference chairman, the publication of the proceedings also falls under the auspices of the Scientific Communications Section. This year seven conferences and workshops, sponsored either completely or jointly, were Sixth Annual Contractors' Conference, Muscle Capillary Basement Membrane Thickness in Prediabetes, Risk-Benefit Assessment of Cyclophosphamide in Renal Disease, Kidney Collaborative Program Conference, Adequacy of Dialysis, Acute Renal Failure Conference, and Prophylaxis and Therapy of Gallstones. Less extensive support, usually along with other Institutes. was contributed to conferences concerning human experimentation, pain, biology of mammalian cells, drug assessment, and an international symposium on poly ADP-ribose.

In addition, the series of meetings on Assessment of the Clinical Trials of Oral Antidiabetic Agents has continued through this past year.

A book entitled "Cystinosis" (Joseph D. Schulman, M.D., editor) was published through the Government Printing Office in April. The authors are primarily either presently or recently on the staff of NIH.

In cooperation with the National Cystic Fibrosis Research Foundation, a comprehensive bibliography is being compiled and indexed. Its arrangement is such that it will be able to be updated from year to year.

In addition, numerous other activities which bear on scientist-to-scientist communication as it affects the Institute are dealt with frequently. Areas where further emphasis should possibly be placed are continually in review.

Project Title: Artificial Kidney Bibliography

Professional Personnel: Mrs. Billie B. Mackey
Dr. Keatha K. Krueger
Dr. N. B. Cummings

Man Years: Total: 0.9
Professional: 0.6
Other: 0.3

Project Description:

The Artificial Kidney Bibliography, now in its seventh volume, is a quarterly recurring bibliography on kidney failure, artificial kidneys and kidney transplantation, which is being produced with the aid of National Library of Medicine's (Medical Literature Analysis and Retrieval System) by the Scientific Communications Section, NIAMDD. It contains about 500 citations per issue with the citations arranged hierarchically to enable similar subjects to appear together. An author index is included with each issue.

The Artificial Kidney Bibliography is made available without cost to National Institutes of Health grantees or contractors who are involved with work related to artificial kidneys, as well as to other government agencies with responsibilities in the area, medical school and hospital libraries. In addition to the free distribution, it is for sale by the Superintendent of Documents, Government Printing Office at a nominal price.

An expansion of this bibliography is underway with plans to include other aspects of relevance to research on artificial kidneys, such as kidney diseases and nephrology. An experimental edition was assembled and a meeting of the Editorial Advisory Committee was held to discuss the experimental edition. Further MEDLARS searches were designed based upon the Editorial Advisory Committee's recommendations. After format revision and inclusion of further suggestions by the Editorial Advisory Committee, the first experimental issue was printed. A meeting of the Committee will be held in June, 1973 to discuss the first experimental issue. Work with the International Committee for Nomenclature and Nosology of Renal Disease to include the International Committee's nomenclature in the bibliography is progressing. Work with the National Library of Medicine's Medical Subject Headings Section is underway to include kidney-related vocabulary in the National Library of Medicine's computerized vocabulary.

Clearance from the Office of Management and Budget to publish the new Kidney Disease and Nephrology Index as a recurring bibliography has been requested.

Project Title:	Diabetes Literature Index
Contract Number:	PH43-67-663
Amount:	$85,239.00
Contractor:	University of Minnesota
Project Officer:	Dr. Keatha K. Krueger
Other Professional Personnel:	Mrs. Billie Mackey
Man Years:	Total: .52 Professional: .32 Other: .20

Project Description:

Diabetes Literature Index, a monthly current-awareness publication of the NIAMDD (now in its eighth volume) was brought about through the cooperative efforts of NIAMDD, NLM, the American Diabetes Association, and three universities (University of Minnesota, University of Rochester, and Case Western Reserve University), as well as a number of individual scientists.

Each month MEDLARS magnetic tapes, which contain all of the current biomedical literature citations used for the preparation of the NLM's Index Medicus, are used for identification of all diabetes-related bibliographic citations. Through 1970 the Diabetes Literature Index has consisted of a keyword in title index and an author index resulting from a computer printout. Starting in 1971, the magnetic tape version of these indexes (prepared at the University of Minnesota) is used to generate copy for offset printing by the Government Printing Office on their Linotron. There are now three formats consisting of a hierarchical subject index of core diabetes literature, an abbreviated keyword in title index of all diabetes-related literature, and an author index including index terms along with the citations (for the first author only). Printing the citations under a preconceived hierarchical structure, which groups related subjects, makes it easier for the user to scan his particular interests in one section.

Diabetes Literature Index is published monthly by the Office of Scientific Communication, NIAMDD, and is available, free upon request, to qualified individuals with NIH grants or contracts who are working in the field of diabetes research, as well as to other government agencies with responsibilities in this area, medical school and hospital libraries. In addition to the free distribution of this publication, it is for sale at a nominal price by the Superintendent of Documents, Government Printing Office.

Project Title: Endocrinology Index

Professional Personnel: Mrs. Billie B. Mackey
Dr. Keatha K. Krueger

Man Years: Total: .7
Professional: .4
Others: .3

Project Description:

The Endocrinology Index, now in its sixth volume, is a bimonthly publication of the NIAMDD, which is produced by the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS). Each issue contains about 4,000 citations of the current world literature in endocrinology. The citations are grouped in eight categories (pituitary, thyroid, etc.) and each category has a preconceived hierarchical organization of subject headings. A list of the index terms assigned to the original article by the indexer appears in the author section. Author and subject indexes appear in each issue. Work was initiated with the Medical Subject Headings Section of the National Library of Medicine for the inclusion of endocrine-related vocabulary.

Liaison was continued with the National Library of Medicine's MEDLARS indexers, the Editorial Office of the Endocrine Society and the publisher, Lippincott, for two endocrinology journals, Endocrinology and Journal of Clinical Endocrinology and Metabolism to be indexed directly from manuscripts by NLM's indexers resulting in faster input of these journals into MEDLARS, and, consequently, into Endocrinology Index.

The Endocrinology Index is available without cost to NIH grantees and contractors in endocrinological work, and to medical libraries. It is also for sale at the Superintendent of Documents, U. S. Government Printing Office at a nominal price.

Project Title: Gastroenterology Abstracts and Citations

Contract Number: NIH 73-2227

Amount: $79,547.00

Contractor: Franklin Institute

Project Officer: Dr. Keatha K. Krueger

Other Professional Personnel: Dr. George Craddock
Mrs. Billie Mackey

Man Years: Total: .25
Professional: .1
Other: .15

Project Description:

In January 1966 the NIAMDD began publication of a current-awareness journal for gastroenterologists. Gastroenterology Abstracts and Citations, now in its seventh volume, appears monthly. Each issue contains approximately 350 abstracts of the most significant gastroenterological literature published in the world and approximately 750 citations covering other relevant gastroenterological literature. The abstracts and citations are divided into three main categories, Pre-clinical Sciences, Diagnostic Procedures and Gastrointestinal Diseases. These categories are subdivided into more specific groupings. Each issue contains a subject and author index and these are cumulated annually.

During 1973, in addition, the contractor is furnishing complete literature coverage on four selected areas, formated on tape suitable for searching, for the benefit of the Digestive Diseases and Nutrition Program of the NIAMDD.

Gastroenterology Abstracts and Citations is available, free upon request, to qualified investigators with NIH grants or contracts in the field of gastroenterology, as well as to other government agencies with responsibilities in this area, medical school and hospital libraries. In addition to the free distribution of this publication, it is for sale at a nominal price by the Superintendent of Documents, U. S. Government Printing Office.

Project Title: Index of Dermatology

Professional Personnel: Mrs. Billie B. Mackey
Dr. Keatha K. Krueger

Man Years: Total: 0.3
Professional: 0.2
Other: 0.1

Project Description:

The Index of Dermatology was established and developed in cooperation with the National Library of Medicine over a period of three years by Universities' Associated for Research and Education in Pathology under a grant from NLM. This grant expired in June, 1971, whereupon five dermatology societies jointly sponsored its publication until March, 1972. To further the NIAMDD Dermatology Program as well as respond to the increased interest and activity in dermatology, NIAMDD undertook the publishing of Index of Dermatology in April, 1972.

The Index of Dermatology is a monthly recurring bibliography on skin diseases, both general and specific, causative and therapeutic agents, and contains aspects such as immunology, mycology, virology, and oral disease. The Index is being produced with the aid of National Library of Medicine's MEDLARS (Medical Literature Analysis and Retrieval System) by the Scientific Communications Office, NIAMDD. It contains about 1200 citations per issue with the citations arranged hierarchically to enable similar subjects to appear together. An author index is included with each issue. An Editorial Advisory Committee of fourteen outstanding scientists working in the area of dermatology has been assembled for continuing the high quality of this publication.

The Index of Dermatology is made available without cost to National Institutes of Health grantees or contractors, who are involved in work related to dermatology, as well as to other government agencies with responsibilities in the area, medical school and hospital libraries. In addition to the free distribution, it is for sale by the Superintendent of Documents, Government Printing Office at a nominal fee.

Project Title:	Artificial Kidney - Chronic Uremia Program Fifth Annual Contractors' Conference Proceedings
Professional Personnel:	Dr. Keatha K. Krueger
Man Years:	Total: 0.1 Professional: 0 Others: 0.1

Project Description:

The Fifth Annual Contractors' Conference, held January 24-26, 1972, brought together about 150 contractors' key staff members with consultants to the Program and Institute staff. The three-day session involved extensive study of existing contract-funded research and development projects with emphasis on program progress and projected plans for the future.

The conferees, in both plenary sessions and intensive individual workshops, communicated research results in the main areas of the Artificial Kidney Program: 1) Hardware Membranes, and Nondialyzer Modes of Therapy; 2) Blood Access and Nonthrombogenic Systems; 3) Clinical and Metabolic Studies. A considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of 62 currently active research and development projects, the Program's consultants and its staff. It is anticipated that this and future conferences will significantly assist attainment of the Artificial Kidney Program's goals--optimum artificial kidney development, improved clinical methodology, and better patient rehabilitation.

The Proceedings of the Conference were compiled and given wide distribution to the community of scientists interested in the field of artificial kidney and chronic uremia.

Project Title: Artificial Kidney - Chronic Uremia Program
Sixth Annual Contractors' Conference and Proceedings

Professional Personnel: Dr. Robert J. Wineman
Dr. Fernando Villarroel
Dr. Keatha K. Krueger
Mrs. Billie B. Mackey

Man Years: Total: 0.4
Professional: 0.2
Others: 0.2

Project Description:

The Sixth Annual Contractors' Conference was held February 14-16, 1973. About 150 participants who were contractors and their staff consultants to the Artificial Kidney - Chronic Uremia Program, and Institute staff attended. The three-day session involved extensive study of existing contract-funded research and development projects with emphasis on intercommunication of program progress and projected plans for the future. The presentations were grouped in three sessions: Hardware, therapy, and clinical and metabolic studies. The conferees in both plenary sessions and intensive individual workshops, communicated research results in the main areas of the Artificial Kidney - Chronic Uremia Program: 1) Biochemistry and Pathophysiology; 2) Therapy and its Evaluation; 3) Nonthrombogenic Surfaces, Cannulae, Membranes and Other Materials. A considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of 67 currently active research and development projects, the Program's consultants and its staff. It is anticipated that this and future conferences will significantly assist attainment of the Artificial Kidney Program's goals--optimum artificial kidney development, improved clinical methodology, and better patient rehabilitation.

The Proceedings of the Conference will be published and distributed in June, 1973.

Project Title: Conference on Adequacy of Dialysis II

Professional Personnel: Dr. Robert Wineman
Dr. Keatha K. Krueger

Man Years: Total: 0.12
Professional: 0.08
Others: 0.04

Project Description:

The purpose of the conference was to bring together a group of qualified and interested persons to discuss the problems of parameters for quantification of dialysis therapy. A new approach to the study of uremia has been proposed based upon the hypothesis that uremic neuropathy may be due to toxins of the molecular weight of 1000-1500 which can be correlated with the clinical picture.

The discussion centered around evaluation and characterization of the solute removed during dialysis, quantification of residual diseased kidney function, and the correlation of these to other problems encountered in patients. The conclusions of this conference were that common data on patients should be collected, therapies manipulated, and successful dialysis therapy characterized. Through these methods toxins and other solutes involved in renal disease can be assessed. The group had a positive attitude toward the feasibility of these methods and the consensus was that this would be a good approach and should yield substantial results.

Project Title: Conference on Acute Renal Failure

Contract Number: NIH 73-C-788

Amount: $19,133.75

Contractor: Downstate Medical Center

Project Officer: Dr. Keatha K. Krueger

Man Years:
Total: 0.13
Professional: 0.06
Others: 0.07

Project Description:

A conference will be held May 18-19, 1973 at Downstate Medical Center, Brooklyn, New York intended to update the understanding of the pathogenesis and treatment of acute renal failure and serve as a forum introduction of new information on its management with dialysis, and prevention in transplant patients. Topics to be covered are pathogenesis, severity and epidemiology, clinical experience, drugs, and acute renal failure following transplantation. Proceedings will be published.

Project Title: Workshop on Kidney Collaborative Program Studies

Personnel: Dr. Nancy B. Cummings
Dr. Keatha K. Krueger

Man Years: Total: 0.22
Professional: 0.20
Other: 0.02

Project Description:

A two-day workshop was organized to provide an exchange of ideas among individuals either at NIH or associated with NIH studies involving collaborative clinical studies, to assess recent information brought out at the recent workshop on Cyclophosphamide and another meeting on anticoagulants, and to promote further discussion of specific details regarding clinical trials including patient selection, baseline information, standardization of pathological techniques and laboratory procedures, and followup procedures.

The presentations covered the basic elements of cooperative studies, coordination of center functions and maintenance of study continuity, data monitoring, research and clinical aspects of cooperative studies, randomization schedules and collection and collation of data, uniform documentation, quality control procedures, choice of treatment, and an overview of cooperative studies, including staff requirements and budgetary considerations. Discussion of ongoing and proposed renal cooperative studies completed the program. There will be no proceedings or report of this workshop.

Project Title: Workshop on Muscle Capillary Basement Membrane Thickness in Prediabetes

Professional Personnel: [Dr. Peter Bennett, Chairman]
Dr. Keatha K. Krueger

Man Years:
Total: 0.08
Professional: 0.04
Others: 0.04

Project Description:

This workshop brought together the major U. S. investigators working on the title area, along with other experts on diabetes and biostatistics, in order to assess results to present, discuss and resolve differences encountered, and propose future action. It was held May 3-4, 1973 in Chicago with 20 scientists in attendance. Generally, the objectives outlined above were met.

Project Title: Prophylaxis and Therapy of Gallstones

Professional Personnel: Dr. Keatha K. Krueger

Man Years: NA

Project Description:

The purpose of the conference was to bring together a group of qualified and interested persons to discuss the status of bile acid therapy and prophylaxis for gallstones. The sessions were arranged in the areas of: 1) Background on abnormalities and physiology involved; 2) Enterohepatic circulation and predictors of stone formation; 3) The metabolic defect; and 4) Therapy and prophylaxis. The state of the knowledge of what causes gallstones and the presently available treatments were the major substance of the conference. Areas suggested for further work included the determination of what causes a stone to nucleate, the mechanism whereby chenodeoxycholic acid causes stones to dissolve, and long-range metabolic results in preventing new stones from forming. Other than a few selected populations such as Indian groups there is no data on heredity and the tendency to form gallstones.

Project Title: Workshop on Risk-Benefit Assessment of Cyclophosphamide in Renal Disease

Amount: $2,450.00 (Purchase Order)

Contractor: National Kidney Foundation

Professional Personnel: Dr. Keatha K. Krueger
Dr. Nancy B. Cummings

Man Years: Total: 0.02
Professional: 0.02
Other: negligible

Project Description:

A workshop to assess the therapeutic index ratio of Cyclophosphamide in patients with renal disease was held in New York City, June 11-13, 1972 under the joint sponsorship of NIAMDD and the National Kidney Foundation.

Clinical data on the effectiveness of Cyclophosphamide in various renal diseases and its toxic effects in both man and experimental animals was reviewed. The participants were then formed into three committees with the following purposes in mind:

1. To assess the present knowledge of the drug and make recommendations concerning the use of Cyclophosphamide in renal disease.

2. To evaluate general toxicity of the drug and make recommendations concerning future studies to add to knowledge concerning effectiveness and toxicity.

3. To evaluate present knowledge concerning gonadal toxicity and to make recommendations concerning present and future evaluations of this problem.

The recommendations of each group will be published in detail. In the meantime a summary of the meeting and these recommendations appeared in the NKF Medical News Report, Volume III, Number 2, October 1972. These recommendations represented a concensus, and are presented as guidelines for physicians who use Cyclophosphamide in the treatment of patients with renal disease.

Project Title: Cystic Fibrosis Bibliography

Contract Number: NIH 72-2214

Amount: $8,965.00

Contractor: National Cystic Fibrosis Research Foundation

Project Officer: Dr. Keatha K. Krueger

Man Years: Total: 0.06
Professional: 0.05
Other: 0.01

Project Description:

This group is preparing and indexing a comprehensive bibliography dealing with the various aspects of cystic fibrosis in order to make available in one place a complete listing of all available references, to establish an initial comprehensive bibliography which can be kept current by annual updating, and to serve as an educational stimulus to individuals in this and allied health fields.

The citations ultimately will be on magnetic tapes suitable for printing via the Linotron. It is expected to be ready for distribution in the fall.

Project Title: Book entitled "Cystinosis"

Professional Personnel: Dr. Keatha K. Krueger

Man Years: Total: 0.10
Professional: 0.08
Other: 0.02

Project Description:

This book, edited by Joseph D. Schulman, M.D., is the only current volume devoted entirely to cystinosis. In addition to Dr. Schulman there were 16 other contributors, primarily individuals now or previously associated with NIH.

In this book are summarized the limited but growing knowledge about this unique dosorder. Hopefully this text will stimulate further investigations of cystine storage disease and will also be relevant and interesting to other scientists studying the biochemistry of disulfides and sulfur amino acids. It should prove valuable to clinician and researcher alike, as clinical information on therapy by conventional means, dietary manipulation, drugs, and renal transplantation is included.

"Cystinosis" (DHEW (NIH) 72-249) is on sale by the Superintendent of Documents (stock number 1745-0060).

Project Title: University Group Diabetes Program (UGDP) - Assessment of the Clinical Trials of Oral Antidiabetic Agents

Contract Number: NIH 177139

Amount: $5,040

Contractor: Yale University

Project Officer: Dr. Keatha K. Krueger (for Dr. Thomas C. Chalmers and Dr. G. Donald Whedon)

Man Years: Total: 0.08
Professional: 0.04
Other: 0.04

Project Description:

A group of biostatisticians has been called together to perform an evaluation of the clinical trials of oral antihyperglycemic agents with reference to their effects on morbidity and mortality in diabetic patients, with particular reference to the vascular complications. The validity and conclusions of the University Group Diabetes Program will be primarily assessed. The report of this group is expected in the fall of 1973.

OFFICE OF THE ASSOCIATE DIRECTOR
FOR
PROGRAM ANALYSIS AND SCIENTIFIC COMMUNICATION

ARTIFICIAL KIDNEY-CHRONIC UREMIA PROGRAM

Summary Report

Introduction

The Annual Reports for the previous years provide detailed background on the problem of end-stage kidney disease and elaborate on developments in the modality for its treatment, chronic intermittent hemodialysis. In addition, these reports give a detailed description of the planned and centrally directed contract program of research and development organized at the National Institute of Arthritis and Metabolic Diseases in 1966--the Artificial Kidney-Chronic Uremia Program--which is aimed at development of improved and less expensive dialysis hardware and methodologies and at optimal rehabilitation of chronic dialysis patients. This background material is not repeated here but the reader might wish to familiarize himself with it by perusing the previous reports.

Current Activities

The Artificial Kidney-Chronic Uremia Program endeavors to bring about improvements in artificial kidney apparatus and methodologies, to increase their effectiveness, to decrease their initial and operational cost, and to improve the rehabilitation of patients treated with chronic intermittent dialysis. Optimal development of new, better dialysis apparatus and methods is still hampered by incomplete knowledge concerning the specifics of the uremic syndrome. Hence, knowledge gained from research into the fundamental aspect of the toxic nature of uremia and basic and clinical studies into the long-term effects of uremia and of chronic intermittent dialysis are critical to arriving at a reasonable solution to the overall problem. Thus, the Program not only sponsors hardward improvement *per se*, but stimulates and supports a wide spectrum of research ranging from fundamental studies designed to clarify the toxic nature of uremia and clinical studies on the complications of chronic uremia and maintenance dialysis, to development of improved dialysis or apparatus. In recent years there has been an increasing emphasis on laboratory and clinical studies intended to bring aobut a greater understanding of the nature of uremia and its major complications.

At present about 65 carefully selected research and development projects are under way through contracts with universities, nonprofit research laboratories and industrial concerns. They constitute a broad approach to the problems of highest priority. Several projects are focused on each of the problem areas and related investigations including development of special adsorbents for uremic toxins, clinical trials of newly developed, more efficient or less costly dialyzers, studies to elucidate the nature of the aberrant metabolic changes in uremia, and the maintenance of a national registry of all patients who are being maintained with the aid of dialysis. The Program is also funding studies aimed at isolation and identification of toxic factors which must be removed from the patients' blood, in addition to metabolic wastes and other substances known to accumulate during kidney failure. Much of the future success in simplifying the treatment of patients with end-stage kidney disease depends on developments in these and other areas of fundamental research.

Accomplishments

In recent years, hemodialysis has become an established and fairly predictable maintenance therapy. A new generation of novel "hollow fiber" artificial kidneys is being developed and one is being produced commercially. The technology of dialysate supply systems has improved considerably in the last six years and there now exist a broad array of devices ranging from highly sophisticated, centrally controlled hospital center systems, to relatively simple self-contained home units.

Long-Term Experience with Peritoneal Dialysis Catheter. Among recent achievements is a report on long-term experience with a new peritoneal dialysis catheter. Peritoneal dialysis is a method of filtering impurities from the blood which utilizes the lining of the abdominal cavity, or peritoneum, as a dialyzing membrane. In 1968, Dr. Heinrich Tenckhoff and associates, working with support from the Institute, developed an indwelling silicone rubber catheter which permitted safe access to the peritoneal cavity for repeated dialyses of patients in chronic (as well as acute) renal failure. Prior to this development it was necessary to puncture the abdominal wall every time a peritoneal dialysis was contemplated, a circumstance which made routine, continually repeated peritoneal dialysis impossible, especially in the home.

Other Developments in Peritoneal Dialysis. Research contractors at the University of Washington are continuing developments in noval peritoneal dialysis equipment. They have already designed and clinically tested a new, fully automated, closed cycle peritoneal dialysis machine which has significantly reduced the cost of treatment because it prepares its own sterile and pyrogen-free dialysate solution. The latter is periodically introduced into the abdominal cavity and emptied therefrom, carrying with it the toxic waste products of metabolism. This equipment has eliminated to a large extent the chances for inadvertent infection. The system prepares sterile pyrogen-free water automatically from tap water through a new technology, "reverse osmosis filtration." Preliminary trials in home dialysis have shown this method to be effective and inexpensive.

With these developments, peritoneal dialysis - at one time almost abandoned in favor of hemodialysis because of a number of drawbacks - has now come to the fore as an acceptable alternative. In fact for some groups of patients it may eventually become the preferred treatment because of special circumstances, including patients who have lost, over the years, all available blood vessel sites for access to their circulation in hemodialysis; also, candidates for renal transplantation who expect surgery within a brief time.

New Style Multiple, Point-Flatplate Dialyzers. In another contract advance, the Western Gear Corporation developed a new type of parallel plate artificial kidney. The new device differs from the widely used Kiil dialyzer only in that closely set rows of tiny, flat-topped pyramids are substituted for the usual parallel grooves on the membrane-supporting surfaces. Clinical tests, comparing the standard Kiil and pyramid-surface types, have revealed that the rate of clearance of a given compound across an artificial kidney membrane is approximately 50 percent greater in the new dialyzer. The data obtained suggested that use of the pyramid-surface dialyzer, rather than the grooved Kiil dialyzer, would permit reduction of dialysis time by approximately one-third. According to an Institute-supported investigator, Dr. James A. Richardson of the University of California, San Francisco, the new dialyzer shortens dialysis time by one-third. These results have been widely confirmed and most manufacturers are now changing their flatplate dialyzers to take advantage of this important advance, which results in a considerable savings of cost and time.

Avoidance of Bone Fractures in Long-Term Kidney Failure from Dialysis. A large percentage of patients with long-term kidney failure who are maintained with the aid of repeated dialysis suffer from a characteristic bone demineralization which weakens the skeleton and makes it prone to fractures. Simultaneously, in many cases, the excess calcium and phosphate liberated from the skeleton is deposited in soft tissues such as the joints, the eye, and even heart muscle. This problem has been subject to very intense research which has borne fruit. This disabling complication now can be prevented.

The bone weakening is the end result of a combination of factors. The progressively decreasing kidney function permits retention in the body of excessive phosphate; at the same time, impaired vitamin D activity prevents adequate intestinal absorption of dietary calcium. As a result, in an effort to increase calcium blood levels to normal, the parathyroid gland secretes excessive amounts of parathyroid hormone which causes a mobilization of calcium (and phosphate) from the bone in an effort to compensate for the low blood calcium levels.

Research workers working individually and in concert have now put together many pieces of the jigsaw puzzle and have proposed novel, clinically effective methods of prevention.

Recent research has demonstrated that the impairment of vitamin D activity in uremia is brought about by the fact that vitamin D itself is not biologically active in the body but a secondary derivative of this vitamin (1,25-DHCC) is. In kidney failure, because of the lack of a normal amount of kidney tissue for this conversion of vitamin D, a profound impairment of calcium metabolism takes place. The Institute has taken steps to procure through contract qualities of 1,25-DHCC for distribution to research scientists and for eventual therapeutic trials. Other research contractors have demonstrated that the overactive parathyroid and calcium abnormalities of chronic renal failure can be avoided in dialyzed patients by restriction of phosphate intake. This is done by adding phosphate-absorbing substances to the diet. At the same time, it has also been found that an elevation of the calcium concentration in the dialysis bath will aid in obtaining higher calcium blood levels and will counteract the otherwise present stimulus for secondary hyperparathyroidism. A combination of both phosphate restriction and blood calcium increase, if started early enough in dialysis, has not been shown to be effective in avoidance of the body dystrophy of chronic renal failure.

Other Innovations. A novel method of blood-vessel access which requires the placing of only one needle into the bloodstream, instead of two as in earlier systems has been studied further. Through a simple valve device, this needle alternately sucks blood from the patient and returns dialyzed blood to his bloodstream. Continuing trials of the device have been successful and patients have expressed a strong preference for it, because it saves half the trauma of placing fairly large-bore needles into their veins three times weekly to connect their circulation to the artificial kidney. It has also been found that, if necessary, the single needle can also be used in a regular large vein without the need for the surgical construction of a subcutaneous fistula.

In addition to the very successful compact Dow hollow-fiber artificial kidney, which is being produced commercially, research is extending the capability of this class of dialyzer. Improvements are being introduced into the basic design through the use of hollow-fibers of a different composition.

Simultaneously, new and successful efforts have started to decrease the time of individual dialysis sessions from the conventional 6 to 7 hours through the use of new dialyzers which incorporate a greater surface area of dialyzing membrane or through the simultaneous use of more than one dialyzer. These approaches are of major significance to the future of dialysis because of their direct impact on the cost of this life-saving treatment.

Working Conferences on Adequacy of Chronic Dialysis

The extension of dialysis coverage to about 90 percent of all workers and their dependents in the United States (the population covered by Social Security Act amendments) will require availability of dialysis facilities and trained technicians. To aid in the search for uniform criteria for evaluation of the adequacy of treatment in chronic dialysis, the Institute is sponsoring a series of conferences on this critical subject.

Assessing the quality of care of dialysis patients is an extremely complex problem. The lack of universally acceptable criteria which can be objectively measured, therefore, results in the reliance upon clinical impressions of the patient's sense of well-being, his ability to lead a productive and enjoyable life, and his freedom from uremic symptoms. The problem is also complicated by the fact that the many clinical dialysis programs utilize different equipment and treatment regimens, with no agreement on optimal treatment. Specialty workshops are planned to seek specific areas for possible research and development of quantitative measures of adequacy.

Guidelines for Evaluation of Artificial Kidneys

With dialyzer development, the Artificial Kidney Program saw need for objective methods to evaluate hemodialyzers. The Program assembled an interdisciplinary group of experts for a conference to establish a "minimal data base" for evaluation of hemodialyzers from early developmental phases to the final phase of long-term patient therapy.

A resultant 84-page document, which has been distributed widely, provides an overall view of presently conceived requirements for an effective dialyzer , outlines measurements for realistic evaluation of the device, and contains nomenclature, appendices, and references to appropriate literature.

Under pending legislation, approval of new artificial kidneys and other critical medical apparatus will be the responsibility of the Food and Drug Administration. Among other things, the new volume was prepared to serve as an objective guideline for evaluation and clearance of any new artificial kidney should this be required by law in the future. All members of of the American Society of Nephrology and the American Society for Artificial Internal Organs have received a copy of the monograph.

Conferences, Publications, and Scientific Communication

In addition to these working conferences, the Institute regularly organizes symposia and workshops on critical problems and opportunities in kidney disease research. These conferences cover topics such as the use of intestinal adsorbents in uremia, blood access devices in dialysis, renal micropuncture, and others. Each year, also, the Institute brings together the research contractors of the Artificial Kidney Program with staff and consultants for a comprehensive meeting at which all ongoing work is reported and discussed. Extensive illustrated proceedings, describing the most recent advances, are published and distributed.

The Institute also distributes to concerned groups and individuals thousands of pamphlets descriptive of kidney disease, dialysis, and kidney transplantation, and responds to inquiries on specific related topics.

Outlook for Kidney Disease and Research and Development

Research conducted and supported by the National Institute of Arthritis and Metabolic Diseases has added much to the understanding and treatment of kidney disease. Investigations such as those discussed in this report have increased our ability to cope with the problems of kidney failure. Additional new research efforts are called for in order that a more widespread and permanent control of kidney disease can be achieved. A meaningful program to reduce kidney diseases must include all of the various components -- research, prevention, treatment, education -- available in our armamentarium. Such a total program must be aimed at all primary kidney diseases as well as at end-stage kidney failure. This combination, led by much-needed research, should yield maximal benefits in the number of lives saved today and the deaths prevented in the years to come.

Contractor: Abcor, Inc.

Amount: $50,446

Title: Development of Capillary Membrane Dialyzer

Objectives:

The objective of this program is the clinical evaluation of a baffled crossflow dialyzer. This unit has been designed to permit more efficient use of dialysate than was possible with a previous radial flow unit.

Major Findings:

The baffled crossflow dialyzer has been under clinical test since July 1972. There have been 67 clinical dialyses with it on 12 different patients. One patient has received 21 consecutive dialyses, being totally supported for more than six weeks. Another has been totally supported for four weeks. These patients plus two more are to be continued until they have six months treatment.

At a blood flow rate of 200 ml/min; clearances and other characteristics of this dialyzer are as follows:

Urea	150 ml/min
Creatinine	110 ml/min
Uric Acid	100 ml/min
Phosphate	65 ml/min
Blood Side Pressure Drop	15-30 mm Hg
Dialysate Side Pressure Drop	40-80 mm Hg
Ultrafiltration Rate	1.35 ml/hr mm Hg
Blood Priming Volume	160 ml

Patient response has been good with stable blood chemistries over the short period covered so far. After a single dialysis, priming volume recovery in tests on six patients averaged more than 90%, with a range from 80-99%. Residual blood volumes after rinse out have been estimated in four tests. With a 300 ml saline rinse, unrecovered whole blood averaged 17 ml; and with a 400 ml saline rinse, it averaged 5.5 ml.

Proposed Course:

This program will be concluded since it has successfully accomplished its objectives.

Contractor: Albany Medical College of Union University

Amount: $72,000

Title: Electronic EEG Frequency Analysis for Evaluation of Uremia

Objectives: The contractor's objective is to attempt to devise an an electronic system which would enable detailed and sophisticated analyses of the EEG in uremia and to attempt to use it as a tool to predict the severity of the clinical status of the patient.

Major Findings: The program has developed the proposed electronic system and has demonstrated that even well dialyzed patients have abnormally slow EEGs when studied in this technique. However, in transplanted patients the abnormal slowing of the EEG was resolved in the first week after successful transplant.

Proposed Course: The investigator plans to apply this technique on patients at other centers to confirm the results he has obtained. Should the results be confirmed, then he expects to simplify the machine so it can be used as a way of judging the clinical state of the patient and the success of therapy.

In addition, he will study why the EEG of some dialyzed patients returns to normal while in others it does not.

Contractor: Amicon Corporation

Amount: $67,200

Title: An Improved Hollow-Fiber Hemodialyzer

Objectives:

The objective of this program is to develop an improved capillary dialyzer utilizing anisotropic polysulfone membranes. The proposed device will have a high ratio of membrane surface area to priming volume and inherently low blood side mass transfer resistance, and will also offer the advantages of autoclavability; higher ultrafiltration rates than achievable with available cellulosic fibres; and a simplified and potentially cheaper manufacturing process since the polysulfone fibres can be potted dry and require no deacetylization or glycerinization.

Major Findings:

During the past year, the dialytic resistivity of the fibers to be used (PMD) has been substantially reduced, most likely as a consequence of an increase in the porosity of the substrate and a decrease in the overall thickness. The polysulfone fibres are a little better than cuprophan for the low molecular weight species and offer a three to four fold reduction in the resistance for bigger compounds such as inulin. The ultrafiltration rates of the PMD fibres in the presence of blood may be controlled within the range of 0.02 to 0.07 ml/min per square meter per mm Hg without substantial variation in dialytic permeability. With a 7 1/2 mil ID, the PMD fibres have burst pressures of greater than 100 PSl. Because of their strength and high modulus, they are not subject to crimping or distortion during the potting and cleaving processes.

Experimental dialyzers have passed extensive toxicity tests by Cutter Laboratories, by Dr. John Autian at the University of Tennessee, and by Dr. Lee Henderson at the University of Pennsylvania. Animal trials were performed by Dr. Lee Henderson who ran *in vivo* tests on 4000 fibre units on 3 dogs and 2 goats.

The *in vivo* performance of a current experimental unit is shown in the following results from a test on a goat in which the blood flow was 75 ml/min, the dialysate flow was 200 mil/min, the area approximately 0.4M, and the unit contained 4000 fibres.

Species	Clearance Ml/min	Resistance (Ro) Min/cm	Calculated Clearance* Ml/min
Urea (BUN)	43	54	108
Creatinine	39	62	98
Inulin	4	900	10

*For 1 M^2 unit, Q_B=200 ml/min; Q_V=500 ml/min.

Proposed Course:

Amicon will continue the engineering development of the unit and scale up to produce a $1.0M^2$ unit for clinical testing, which will be done by Dr. Henderson of the University of Pennsylvania.

Contractor: Avco Everett Research Laboratory

Amount: $93,310

Title: Blood Flow Considerations to Minimize Thrombosis in Artificial Kidney Systems

Objectives:

Investigation of hydromechanical phenomena and surface materials properties relevant to initiation and propagation of thrombus formation.

Major Findings:

Work to date includes an investigation thrombus formation in separated flows and has led to the study by direct observation of the formation and growth of unattached platelet aggregates and their embolization from a separated flow region. Studies have also been made of the formation of attached or surface aggregation within the separated flow regions. Such aggregation occurs under differing flow conditions from that in which floating aggregates form.

Proposed Course:

1. Investigate blood/surface interfacial phenomena under controlled flow conditions as a function of systematically controlled surface properties of polyurethane test surfaces and systemic anticoagulation.

2. Investigate the influence of locally infused thrombus inhibiting agents as a means of controlling or eliminating thrombus formation in shunts or dialyzers.

3. Develop additional clinical applications of this theoretical work.

Contractor: Avco Everett Company

Amount: $109,801

Title: Development of Avcothane Cannulas

Objective:

Utilizing the proprietary substance Avcothane, these people proposed to develop a cannula of this Avcothane as well as to investigate various causes for recurrent thrombosis with the use of cannulas in hemodialysis.

Major Findings:

The process for coating cannulae with an Avcothane that retains its nonthrombogenic properties has been established. Implantation of both silastic and Avcothane cannulae has been begun. The goat was selected as the animal model as a standardized breed has become available which reduces a great deal of animal to animal variation. In this model, an increasing white blood cell count and a decreasing platelet count have proven to be harbingers of clotting of the shunt.

Proposed Course:

Completion of *in vivo* animal testing and comparison of function of silastic and Avcothane cannulae.

Contractor: Battelle Memorial Institute

Amount: $119,700

Title: Feasibility of Microencapsulated Detoxicants for Removal of Metabolites Via Ingestion

Objectives:

The overall objectives of this contract are the development of microcapsules capable of irreversibly binding urea, ammonia and other toxic metabolites and the evaluation of these capsules for gut dialysis.

Major Findings:

The Battelle group have found that chemical binders of urea are inadequate and the most rewarding area of investigation is to develop methods to stabilize urease and bind this enzyme to polymers which can then be encapsulated. The urea would then react with the urease and aspartase and be converted to ammonia which then could be adsorbed by resins containing sulfonic acid groups which are also encapsulated. The stabilized urease has been encapsulated as well as the resins contining sulfonic acid. Their microencapsulated system has shown to be effective in vitro and in azotemic animals. The concentrations of selective nitrogenous metabolites in various regions of the dog small intestine were determined in control and azotemic animals and correlated to blood levels of creatinine urea, and uric acid.

Proposed Course:

They are presently planning to test their microcapsules in animals. They plan to study the physiology of the GI fluids and the behavior of those fluids with their microcapsules.

Contractor: Brooklyn Veterans Administration Hospital

Amount: $15,000

Title: Identification of Proteins Interacting at Plasma/Biomaterial Interfaces

Objectives:

To identify the proteins deposited by plasma, both in the presence of and in the absence of an air interface, onto materials considered for or related to use in artificial kidney membranes and cannulae. The findings of this work should unravel mechanisms of some of the rapid initial events which occur when blood enters an artificial kidney system that may later rule the fate of blood in the device (i.e., plugging) and in the patient (i.e., loss of platelets and of certain plasma proteins).

Major Findings:

1. Using the recording ellipsometer, it has been shown that intact plasma deposited onto 7S gamma-globulins that had been adsorbed on quartz-like surface, and then the plasma removed a large amount of matter. The deposits did not react with anti-human fibrinogen serum. Deposits left by factor XII deficient or activated normal plasma, or by serum on the globulin films, were also removed by such intact normal plasmas. If small amounts of fibrinogen were present in the globulin films, much less matter was deposited by subsequent exposure to normal plasma. Since plasma does deposit both fibrinogen and 7S gamma-globulins on a variety of surfaces, these findings suggest complex interactions determine what a platelet in its native plasma will see as their outcome, and thus whether or not it will adhere and undergo metamorphosis.

2. Sites of interaction among antigen that had been adsorbed onto a flat surface, and matching antisera can be detected not only by ellipsometry, or with water vapor condensation or interference colors as found in previous years, but also by staining with Coomassie Brilliant Blue R. Preliminary experiments indicate that this method can be applied even to prewetted Cuprophan tubing and will allow identification of proteins adsorbed out of blood.

3. Investigation of the behavior of pre- and post-dialysis plasma samples has been studied. Thus far, (1) the partial thromboplastin times in glass and polystyrene, the ability (2) to deposit matter on glass that corrected the clotting of factor XII deficient plasma, and (3) to deposit and then convert fibrinogen and 7S gamma-globulins, all appeared normal. Like normal plasma, these samples upon activation lost the ability to convert pre-adsorbed fibrinogen but remained partially able to convert the fibrinogen that the plasma itself had deposited.

Proposed Course:

Current studies noted above will be extended and emphasis will be placed upon applying techniques of Dr. Vroman's laboratory to uremic, pre- and post-dialysis sera; membranes pre- and post-dialysis; and with any new biomaterials for artificial kidney for which the Artificial Kidney - Chronic Uremia Program needs an evaluation of thrombogenicity.

Contractor: University of California

Amount: $89,000

Title: Nitrogen Metabolism in Uremia

Objectives:

To study the alterations in normal amino acid metabolism in uremia. These studies are to include tissue sample analysis to give an accurate representation of amino acid pool fluxes.

Major Findings:

The investigator has demonstrated that histamine is an essential amino acid to uremics and also to normal subjects. The investigator's other results so far are most consistent with the concept that uremia is a starvation state. Most of his findings in uremic patients are consistent with those findings in normal patients on severely restricted or starvation diets.

Proposed Course:

The investigator will continue this course since his data is scanty so far. He will expand his data base to a statistically significant level.

Contractor: Case Western Reserve University

Amount; $60,000

Title: Application of Laser Doppler Techniques to Artificial Kidney Design

Objectives:

To apply the laser doppler flowmeter to study the kinetics of blood coagulation.

Major Finding:

1. Derivation of general relationship between the space - time correlation function and the observed spectrum from the laser doppler velocimeter.
2. _In situ_ measurements of rate of formation of fibrin from fibrinogen.
3. Development and testing of techniques for determining degree of polydispersity of an arbitrary dispersion from the laser doppler signal.

Proposed Course:

1. Develop and check computational techniques which relate flow patterns near membranes to mass transfer rates.
2. _In situ_ measurement of fibrinogen formation and platelet agglutination.
3. Develop techniques for experimentally determining degree of polydispersity within the flowing medium.

Contractor: Cedars-Sinai Medical Center

Amount: $125,000

Title: Osteodystrophy and Divalent Ions in Kidney Failure

Objectives: Perform a systematic and comprehensive study to evaluate the mechanism leading to deranged divalent ion metabolism in renal failure.

Major Findings: Evaluations of mechanisms involved in the development of abnormalities of divalent ion metabolism in 1) stable chronic renal failure patients; 2) patients undergoing chromic hemodialysis; 3) patients from post-renal homotransplantation; and 4) patients with early minimal degrees of renal failure in approximately twenty patients per group. Investigation into the role of Vitamin D resistance and altered parathormone activity in producing the skeletal and soft tissue pathology indicates that a progressive Vitamin D resistance occurs and skeletal unresponsiveness to parathormone in conjunction either initiates or aggravates the osseous and soft tissue abnormalities.

In addition the investigators have found higher levels of skin calcium in patients dialyzed with higher dialysate calcium concentrations, although phosphate intake was uncontrolled.

Proposed Course: Continue investigations into the mechanisms of divalent ion metabolism in uremic osteodystrophy with application of findings toward rational modes of therapy for this disorder.

Cedars-Sinai Medical Center, Los Angeles, California

Amount: $43,000

Title: The Dialysis Disequilibrium Syndrome

Objective:

To elucidate the cause for the so-called "dialysis disequilibrium syndrome."

Major Findings:

The investigator has discovered that the dialysis disequilibrium syndrome most likely is due to cerebral edema. He has demonstrated that this edema is not on a base of urea, glucose or electrolyte fluxes. He has presented evidence consistent with the concept of the development of the "idiogenic osmoles" intracellularly in the cerebral cortex.

Proposed Course:

The investigator will attempt to characterize these idiogenic osmoles. Specifically, he will measure intercellular brain PH on the supposition that these idiogenic osmoles are hydrogen ions released from protein during alterations during inter- and extracellular PH.

Contractor: Celanese Research Company

Amount: $120,000

Objectives: Explore cellulose acetate membrane variants in an effort to obtain optimized hemodialysis membranes.

Findings: A demonstration run has produced enough membrane for clinical testing. This membrane has transport properties essentially similar to one developed for the Program by the Envirogenics Company.

Proposed Course: Further work will be done to optimize cellulose acetate membranes, and a small exploratory effort will examine electret membranes, PBI membranes, and composite structures.

Contractor: Cleveland Clinic

Amount: $67,000

Title: Development of an Envelope Kidney

Objectives:

The objective is to develop a sterile package of a supported tubular membrane with blood ports attached so that a patient can wind a coil dialyzer at a significant cost reduction.

Major Findings:

Components of the Coiled Envelope Kidney were redesigned and constructed. The assembly process was simplified and the winding of the coils was done with a winding machine designed to choose and monitor the winding tension. Using our standardized test protocol coils were tested and data reduced on our computer program. Initial units made with 0.85 m^2 surface area were compared with commercially available coils for fluid dynamics and mass transfer and shown to be comparable. A controlled study of 1.0 m^2 surface area units was then made to study the effect of strip tension (4-35 pounds) on coil characteristics and performance at a uniform simulated blood flow rate of 200 ml/min and varying dialysate flow of 1-16 liters/min at single pass. Blood side pressure drop showed a direct correlation with strip tension. Calculated blood compartment volume was relatively low being about 160 ml/m^2 at the lowest strip tension and 125 ml/m^2 at the highest strip tension. Mass transfer of urea, creatinine, and uric acid was studied and indicated membrane limitation over 9 liters/min. At these flows, clearances increased from 130 to 170 ml/min for urea and 105 to 130 ml/min for creatinine with increasing tension. Uric acid clearance ranged from 55 to 90 ml/min with less dependency on strip tension. Likewise, mass transfer coefficients were shown to increase 75% for urea, 30% for creatinine, and 65% for uric acid from the lowest to highest performing unit. Water removal averaged a pound per hour.

Dog dialyses were carried out with these laboratory made components and indicated no particular problems related to the design. The blood port design was then tooled for mass production and initial ports were molded. An improved version of the central core was fabricated. A patient model of the winding machine was constructed. A strength study of available rigid nets for use as a primary support was carried out and one net was selected for further studies. Present work is being directed to optimize the construction of the Coiled Envelope Kidney and to characterize it in vitro. Dog dialyses will then be carried out prior to clinical trials. Present estimates of the cost of the Coiled Envelope indicate that it can be manufactured at a cost which would reduce the cost of coil maintenance dialysis.

Proposed Course:

This contract will be continued to conclude the development of the coil envelope kidney.

Contractor: The Cleveland Clinic Foundation

Amount: $47,000

Title: Development of New Concepts in Blood Access Devices

Objectives:

It is the objective of this program to develop new methods for blood access devices for hemodialysis having a minimum of complications.

Major Findings:

Natural tissue subjected to "biolized" treatment was utilized. This treatment as evidenced by the long-term thromboresistance of similar materials treated and used in cardiac prostheses warrants their much more thorough investigation as a possible material for use in hemodialysis blood access devices. In this case "biolized" treatment consisted of taking fresh bovine mesenteric arteries and inactivating the material for use by treatment with aldehyde. The material was then used in the construction of devices for implantation.

Three methods of blood access are being studied: 1) a puncturable subcutaneous shunt (modified Cimino-Brescia fistula), 2) a puncturable extracutaneous shunt, and 3) a detachable extracutaneous shunt (modified Quinton-Scribner shunt). All shunts are designed to be connected to the vascular system through end-to-end anastomosis with the aid of Dacron fabric, and to have blood contracting only a single surface, the "biolized" surface. The outer surface of the shunt body is coated with triple centrifuged purified natural rubber for reinforcement and to provide self-sealing properties for the puncturable shunts.

Replacement of a renal artery with a biolized graft was carried out in four dogs. A contralateral nephrectomy or ligation of the contralateral renal artery was done in all animals and the patency of the graft studied. Grafts 1.5 to 2.0 cm in length with internal diameter of less than 3.5 mm have remained patent in three animals for periods of 3, 10, and 12 months. Puncturable subcutaneous grafts of 10-18 cm long with internal diameter of less than 3.5 mm were implanted in four dogs between the carotid artery and jugular vein. Patency of the grafts ranged from 12 to 23 days with an average of 17 days. The maximum number of punctures per shunt using a 14 gauge needle was two. Three dogs were implanted with puncturable extracutaneous shunts 6-15 cm long with internal diameter of less than 3.5 mm. Patency of these grafts ranged from 10-14 days with an average of 12 days. The maximum number of punctures per shunt using a 14 gauge needle was four. Macroscopically tissue reactions to natural rubber appeared minimal and a very thin pseudoneointima was noticeable inside the shunt.

As a control study seven similar silicone rubber shunts commercially available and cut to a length of 14 cm were implanted between the carotid artery and jugular vein in dogs and showed a patency of 2-10 days with an average patency of 5 days.

Proposed Course:

This contract will be continued for further evaluation of these concepts in animals.

Contractor: Columbia University

Amount: $45,000

Title: Identification of a Nonthrombogenic Environment

Objectives:

Identify those characteristics of surfaces and blood which make an atraumatic nonthrombogenic environment possible.

Major Findings:

A Captured Vortex Device has been used for *in vivo* testing of artificial materials under controlled blood velocities. Particular parts of the device are isolated from contact with possibly damaged intima and consequent release of tissue thromboplastin. The role of various surfaces, turbulent flow, platelets, coagulation factors, temperature, and blood velocity on the initiation of clotting are being investigated. Various histologic chemical and physical manifestions of clotting are being measured. The effects of aspirin and other drugs on clotting are also being studied.

Proposed Course:

Employ the captured vortex device in a program of materials evaluation.

Contractor: Dow Chemical U.S.A. and University of California

Amount: $209,916

Title: Evaluation of Experimental High Flux Artificial Kidneys

Objectives:

Develop and clinically evaluate hollow fiber kidneys with improved clearance for higher molecular weight solutes.

Major Findings:

The molecular etiology of the morbidity associated with chronic dialysis therapy is poorly understood at present. There is, however, an increasing body of evidence to indicate that solutes above molecular weight 200 - those with low clearance in current dialyses - may be an important factor in this syndrome.

In an attempt to more clearly define the role played by differing molecular weight species, high flux hollow fiber kidneys have been developed that have clearances of 40 ml/min. in the 5000 M.W. range. By adjusting the dialysate flow rate with these units, the value of the mass transfer coefficient of differing solutes can be altered to produce clearance relationships that will allow for studies of specific molecular weight ranges.

The urea, creatinine, raffinose, Vitamin B_{12} and inulin clearance are 185, 163, 105, 78 and 50 ml/min. respectively at Q_B 200 ml/min. Q_D 500 ml/min. It is possible with these units to preferentially increase the higher molecular weight clearance while leaving the lower molecular weight solute clearance at levels comparable with 1 M^2 units by varying the dialysate flow. Using the combination of flow and membrane properties, the effect of high clearances of specific molecular weight ranges can be studied while the remaining solutes are cleared at normal or lower rates.

These 2 M^2 high flux cellulose acetate kidneys have been used clinically for more than two months. Ultrafiltration in these units was over ten times that found in the 1 M^2 cellulose hollow fiber kidney but not problems were encountered in clinical use with the pressure balancer and the clinical therapy with this high flux kidney was well tolerated.

Proposed Course:

This important investigation of the clinical performance of high flux dialyzers will continue.

Contractor: Envirogenics Company (Aerojet-General Corporation)

Amount: 0

Title: Research and Development of Hemodialysis Membranes

Objectives: To produce sufficient quantities of cellulose acetate membranes so they can be distributed to dialysis centers in the United States for clinical trials.

Proposed Course: Termination

Contractor: Esso Research and Engineering Company

Amount: $66,000

Title: Liquid Membrane Capsule System for the Treatment of Chronic Uremia

Objectives:

The liquid membrane concept, invented at Esso Research, is thought by many to be one of the few new membrane concepts of the last several decades. Liquid membrane capsules (LMC) are emulsion sized (10 to 100 microns in diameter) thin spherical liquid shells that can be used for encapsulation. In the application of liquid membrane to chronic uremia, reagents would be encapsulated in liquid membranes. The toxins of uremia would transfer through the liquid membranes into the reagent and be trapped, thus preventing toxin return to the patient. Exploratory work has been done to establish the feasibility of liquid membrane encapsulated reagent systems for removing some identified uremia toxins.

The primary goals of the coordinated Esso Research and Engineering Company and the University of Pennsylvania program for the first year are:

1) Development of effective liquid membrane formulations for the removal of urea and phosphate ion.

2) Partial elucidation of the rate of transfer of the above uremic toxins across the gut wall.

3) Demonstration of improved blood chemistry of uremic rats fed LMC.

Contractor: Franklin Hospital, San Francisco, California

Amount: $25,940

Title: Permeability of High Flux Operational Dialyzers with Variable Support Geometry

Objectives:

The investigator is to study the permeability of five (5) new membranes to water and to small and middle molecular weight solutes in two existing clinical dialyzers - a standard Kiil and a multiple point support Dy Kiil. These membranes have previously been characterized in laboratory test cells.

Contractor: Franklin Institute Research Laboratories

Amount: $00.00

Title: Hydrogel Graft Coated Plastic Parts for Hemodialysis

Objective:

The objective of this program is to fabricate and clinically evaluate hydrogel-graft-coated plastic cannula tubing, tips and connectors with improved blood-compatibility.

Major Findings:

Using standard designs, tips and connectors are made from a stiff polyurethane 3-425-1-x and the tubing from a flexible polyurethane 3-100/425-1-x (70/30) by the Stanford Research Institute. A thin coating of hydrogel is to be grafted to the blood exposed surfaces. The hydrogels being investigated are poly(2-hydroxyethylmethacrylate) and poly(acrylamide). These materials have recently been found to have promising blood-compatibility properties in our and other heart-assist device development programs supported by NHLI.

The grafting method consists of generating free radicals on the plastic substrate surface by exposure to atomized hydrogen produced by microwave excitation at ca. 3 torr in a mixture of hydrogen and helium, and contacting the activated surface with the hydrogel-forming monomer for simultaneous covalent bonding and conversion of the monomer to hydrogel. Flexible hydrophilic coating are obtained which contain ca. 40% wt. % water in the case of poly (2-hydroxyethylmethacrylate and 40 wt. % water in the case of poly(acrylamide). The coated products can be sterilized by autoclaving.

Proposed Course:

This 30 month contract will continue and is expected to accomplish its objectives.

Contractor: Georgetown University

Amount: $61,629

Title: New Approaches to Design of Chronic Dialysis Cannulae for the Prevention of Infection

Objectives:

1. Develop a method of fixation of an AV cannula to minimize turbulence related thrombosis in cannula dislodgement due to vessel necrosis.

2. Develop an antithrombogenic material for use in the AV cannula.

3. Prevent cannula sinus tract formation by means of developing a percutaneous seal for the skin prosthetic junction.

Major Findings:

The final design of the shunt for clinical trials has been determined. This design has several new improvements: an oblique teflon velour tip with an end-to-end anastamosis; an extremely short protruding segment above the skin; an improved method of hepacone G coating; a skin button markedly reduced in thickness; a lessened distance from the site of insertion of the shunt into the vessel to the emergence from the skin.

Proposed Course:

Continue present objectives with clinical trials of approximately 60 of the new shunts.

Contractor: Gulf South Research Institute

Amount: $140,594

Title: A Study of Synthetic Polypeptides as Possible Hemodialysis Membranes

Objectives: To produce a polypeptide (poly- -methyl-D-glutamate) membrane for an in vivo clinical evaluation study.

Major Findings:

This membrane is prepared in a dry form; it has excellent blood compatibility; it has excellent mechanical properties; and it has a promising pattern of permeabilities, especially for higher molecular weight solutes.

Membrane transport properties obtained in the laboratory have been compared to preclinical and clinical data obtained using several commercial dialyzers. There is an excellent correlation of membrane ultrafiltration rates obtained under these various conditions. The data indicate that the higher the ultrafiltration rate the greater the difference between dialysate and blood filtration rates. The data become difficult to interpret also, due to the non-linear response observed for high ultrafiltration membranes such as cellulose acetate (approx. 8 to 10 times the rate of cuprophane). These data indicate that for the low molecular weight species, urea and creatinine, the theoretical transport as measured in the laboratory is not captured in the present commercial dialyzer but the ranking orders are maintained. Higher molecular weight species are currently being evaluated.

Clinical trials have been initiated. It is anticipated that approximately five patients will be evaluated over a minimum of four to six months to test the effectiveness of polypeptides membranes in hemodialysis.

Proposed Course:

The program will be continued to solve storage and other membrane production problems, to prepare membrane for clinical trial and to carry on the trials for up to six patients for six month periods.

Contractor: Gulf South Research Institute

Amount: $29,602

Objectives:

Serve as a facility for the evaluation of transport and mechanical properties of potential hemodialysis membranes.

Findings:

During the first year of the project four flat sheet membranes have been studied.

Proposed Course:

Continue to apply a consistent set of testing methods to generate data by which membranes can meaningfully be compared.

Contractor: Jewish Hospital of St. Louis

Amount: $49,760

Title: Bone Metabolism in the Chronic Uremic State and its Response to Vitamin D and Diphosphonate Therapy

Objectives:

The use of a rat model of chronic uremia in which to study bone and collagen metabolism in uremia and to study the effect of diphosphonate, Vitamin D_3 and 25-hydroxycholecalciferol therapy on the altered bone and collagen metabolism in the uremic model.

Major Findings:

This contract is an investigation of the hormonal regulation of bone metabolism and calcium transport in the chronic uremic state. It has been demonstrated that in chronic uremia there is an increase in soluble immature collagen fragments in bone and a decrease in the more mature bone collagen. There appears to be a block in the maturation process, as well as an increased degradation of mature collagen. In addition 2 calcium-binding proteins have been isolated from human intestine. It was also shown that hypomagnesia does not affect certain skeletal responses to PTH and 3'5' AMP; that there is an overall decrease in glucose uptake by uremic bone; and that control over intestinal calcium transport exerted by either PTH or Vitamin D_3 is not mediated via cyclic nucleotide activity.

Proposed Course:

The investigator will study the effect of diphosphonates, Vitamin D_3 and 25-hydroxycholecalciferol, in chronically uremic rats with the appropriate controls, on bone and collagen metabolism.

Contractor: The Johns Hopkins University

Amount: $46,000

Title: Metabolic Studies in Uremia

Objectives:

To study: a) the effects of posture, changes in extracellular fluid volume and plasma potassium, on plasma aldosterone levels in anephric patients on hemodialysis; b) carbohydrate intolerance in uremia; and c) plasma cortisol levels during dialysis.

Major Findings:

1. Dialysis studies reveal a prolonged cortisol half-life, varying between 60 and 185 minutes in 6 studies. Cortisol secretion occurred in episodic bursts usually lasting 15 minutes or less. The frequency of these episodic bursts of cortisol secretion was remarkably constant, an average of 5 secretory episodes occurring during the 4 hour study.

2. The studies on carbohydrate intolerance have been extended to include studies on 27 subjects with chronic uremia, performed prior to entry into the dialysis program and again after becoming stabilized on the chronic hemodialysis regimen. The analyses completed to date on the 54 studies in 27 uremic individuals identify a range in the degree of carbohydrate intolerance as judged by the glucose disappearance assimilation rate (K_G). The mean value for fasting growth hormone was significantly higher in the patients prior to entering dialysis than when re-studied while on intermittent hemodialysis. In the pre-dialysis studies, intravenous administration of glucose was associated with a twofold rise in immunoreactive growth hormone. This abnormal response was markedly diminished by hemodialysis, though not completely abolished. Mean values for immunoreactive insulin were unchanged following dialysis. The two changes identified are a reduction in the fasting level of growth hormone and in the magnitude of growth hormone response to intravenous glucose and an increase in the K_G value, and are significant since they represent paired data obtained in 27 subjects before and after beginning hemodialysis.

3. Other abnormalities identified include marked elevation of levels of renin, angiotensin and aldosterone prior to nephrectomy, a variable and usually marked prolongation of cortisol half-life, persistent hyperinsulinism in response to glucose administration, marked elevation in fasting growth hormone levels with a paradoxical rise in response to glucose administration and an abnormal carbohydrate tolerance plus abnormalities in fatty acid concentrations in plasma.

Proposed Course:

Terminated

Contractor: Karolinska Institutet and S:T Eriks Sjukhus

Amount: $15,750

Title: A Study of Uremic Toxicology

Objectives:

Through molecular weight fractionation and other analytical procedures as well as dialysate sorbent technology, the contractor is attempting to separate, identify and assay potential uremic toxins in various biologic fluids including plasma, urine and dialysate collected under specific clinical and therapeutic conditons.

Major Findings:

A new high speed gel filtration method which provides a separation technique based on the important solute characteristic of molecular size and weight has been developed. With this technique the contractor has been able to define readily detected differences between normal and uremic plasma in their patterns of ultraviolet absorbing constituents. The abnormal components of uremic plasma have been detected in both peritoneal and hemodialysate. Fractional separations of these abnormal uremic solutes based upon molecular size and weight differences have been made and these are currently undergoing further chemical analysis utilizng techniques of amino acid analysis and gas chromatography.

Ten such peaks have been defined and have revealed significant differences when normal, untreated uremic and dialyzed uremic plasma are compared to one another. A qualitative identity between untreated uremic plasma and normal urine has been detected. Identification of potential uremic deficiency states has been ascertained by finding one peak quantitatively smaller in the uremic as compared to the chronically dialyzed patient and more so to the normal patient.

Proposed Course:

This contract will be continued for at least six months to determine whether the techniques have utility in obtaining useful data on the problem of abnormal uremic metabolites.

Contractor: Louisiana State University at New Orleans

Amount: $35,083

Objectives: Develop solid surface fluorescence techniques for uremic "toxins" and intermediate metabolites of interest in the study of uremic metabolism.

Major Findings: Methods have been developed for tryptophan, uric acid, and transketolase

Proposed Course: The emphasis of the program will remain on "toxins" and normal metabolic intermediates.

Contractor: Mayo Foundation

Amount: $88,000.

Title: "Uremic Polyneuropathy: (a) Instrumentation and Quantitation of Sensation as an Index of the Effectiveness of Dialysis and, (b) Histologic and Lipid Evaluation of Biopsied Nerve".

Objective: Using instruments he has developed the contractor will:

(1) Test the usefulness of these new instruments in detecting the presence of mild uremic neuropathy by comparing them to currently available methods.

(2) Use these quantitative measurements as an index of the severity of neuropathy in patients treated by a standard dialysis regimen and those on infrequent dialysis.

(3) Use histologic and biochemical studies of nerve and liver tissue to elucidate pathogenic mechanisms in the development of uremic neuropathy.

Major Findings: To date the investigator has completed the instrument development and standardization on normal subjects.

Proposed Course: To continue work on his objective.

Contractor: Mayo Foundation

Amount: $130,444

Title: Value of Maintaining Parathyroid Hormone Suppressive Calcemia in Prevention of Bone Disease and Abnormalities in Calcium Homeostasis in Patients on Long-Term Hemodialysis

Objectives:

This project is directed toward assessing the value of maintaining physiologic plasma concentrations of both calcium and phosphate in suppressing parathyroid hormone secretion and preventing metabolic bone disease and abnormalities of calcium homeostasis in patients maintained on long-term hemodialysis. Parathormone assays in relationship to varying levels of dialysate calcium, dietary calcium, and possible vitamin D supplementation will be studied. Parathormone assays in relationship to phosphate which had been lowered by oral administration of aluminum carbonate and hydroxide will also be studied. Bone biopsies and bone density studies will be performed.

Major Findings:

Thus far, the dialysate Ca level has been identified as a critical factor in the pathogenesis of renal osteodystrophy. In addition, it has been found that maintenance of serum phosphate near normal levels with phosphate-binding gels is crucial. Serum PTH levels respond to changes in these critical factors.

Proposed Course:

Evaluation of dialysate Ca concentration of 8 mg%, its effect on PTH, and follow-up of patients receiving renal transplantation.

Contractor: Mayo Foundation

Amount: $39,085

Title: Metabolism of Creatinine and Guanidine Compounds

Objectives:

To define the metabolic abnormalities of nitrogen metabolism in the uremic state with the ultimate goal to design controls that might by-pass the abnormalities induced in uremia. To approach this aim, initially, by evaluating the extent of creatinine catabolism in the gut and creatinine catabolite formation in chronic renal failure; and to determine the metabolic significance of the guanidines in chronic renal failure.

Major Findings:

$^{14}C_3$-creatinine has been given IV to two patients with stable chronic renal failure and to two anephric patients maintained on chronic hemodialysis while awaiting renal transplantation; and orally to one patient with stable chronic renal failure. Analyses for ^{14}C were made upon all urine and feces, blood, and expired respiratory gases collected at intervals; which analyses included ^{14}C appearing in creatinine metabolites (methyl amine, sarcosine, methyl hydantoin). It has been shown that: 1) the apparent creatinine deficit in a patient with reduced renal function was not secondary to a decreased rate of formation; 2) the creatinine was metabolized to sarcosin, methyl amine and methyl hydantoin probably by the GI tract microflora.

Proposed Course:

1) Measurement of creatinine catabolism in the gastro-intestinal tract of control and of anuric man via creatinine balance studies, using creatinine-$^{14}C_3$, creatinine 1-^{14}C, or creatinine 3-^{14}C.

2) Determination of guanidines (monosubstituted guanidines, creatine, creatine phosphate, methylamine and demethylamine) will be made and correlated with severity of uremic symptomatology at different levels of uremia, pre- and post-dialysis.

3) Experiments to test the clinical impression that meat contains or give rise to a substance(s) not present in milk or eggs that is toxic for uremic patients are planned for 3 patients with creatinine clearances of 3-8 ml/min over four study periods: (1) adjustment; (2) animal protein source as eggs and milk, only; (3) meat as major dietary protein; and (4) adjusted eggs/milk until levels for meat diet symptomatology reached to total 12-14 weeks.

Contractor: Minneapolis Medical Research Foundation

Amount: $130,346

Title: Computer Data Bank to Assess Adequacy of Dialysis

Objectives: To utilize their computerized data bank of clinical data on dialysis patients to assess the feasibility of this approach to the quantitation of adequacy of dialysis.

Major Findings: A low-flow dialysate study was designed to test the "middle molecule" hypothesis in a relatively large group of patients in a controlled situation making use of the existing computerized data bank on dialysis patients maintained by this organization. This study is now underway. Retrospective and prospective studies on endotoxin in febrile reactions during dialysis led the investigators to conclude that these febrile reactions are usually associated with the presence of circulating endotoxemia in the absence of culturable bacteria. The probable source of the endotoxin is the dialysate. In addition, retrospective studies designed to test the computerized data system were performed, and improvements made in the system as a result.

Proposed Course: The low-flow dialysate study, already underway, will be completed and the data analyzed. The effect of anabolic steroids on anemia of chronic dialysis patients will also be studied. Results will be statistically analyzed to determine which factors influence response to therapy. In addition, further refinements will be made in their clinical and research applications of the computerized medical data system for dialysis patients.

Contractor: University of Minnesota

Amount: $46,648.00

Title: Fluid Dynamics of Blood Cells

Objectives:

This is a new contract with six objectives:

1. Analysis of blood velocity profiles in narrow channels using a 3-D microscope system.
2. Study of hematocrit skimming layers using fluid conductivity measurements.
3. Couette skimming layer force evaluation.
4. Analysis of flow enhanced diffusion coefficients using the Taylor Dispersion Method.
5. Studies of formed element behavior following wall contact using electron and phase microscopy.
6. Construct and test a novel blood access device.

Contractor: University of Missouri

Amount: $29,000

Title: Adherence of Blood Components to Artificial Kidney Membranes - Effects on Dialysance of Low and "Middle" Weight Solutes

Objectives:

To study the effects of hemoperfusion on dialysis membrane performance. The purpose of this is to draw conclusions as regards the potential of different membranes for reuse and possible pitfalls involved in membrane reuse.

Major Findings:

To date the investigator has demonstrated marked decreases in membrane permeability to a variety of solutes - electrolytes and organic following the initial hemoperfusion. He has also demonstrated that after the initial perfusion the increment of decrease in solute permeability with each dialysis is considerably less than it is after the first dialysis. There has been some investigation of various rinsing techniques and demonstration that an air rinse greatly decreases coil clearance to the point of making coil reuse impractical. It would, therefore, be essential to utilize other rinses (such as saline) if one is going to reuse the coil.

Proposed Course:

To continue characterization of a variety of membranes in the coming year to include the Dow Hollow Fiber.

Contractor: Montreal General Hospital

Amount: $63,019

Title: Determination of Nutritional Requirements and the Study of Body Composition in Patients on Chronic Hemodialysis.

Objectives: Dr. Kay's objectives are to determine the nutritional requirements and to study the body composition of patients on chronic hemodialysis.

Major Findings: The investigator has demonstrated that renal osteodystrophy may respond well to dihydrotachysterol (DHT) and he has successfully reversed progressive bone disease in dialyzed patients studied thus far. He has also studied fluoride levels in patients undergoing dialysis in both fluoridated and non-fluoridated areas and has found no difference in the degree and type of bone disease in these two populations.

In addition he has undertaken preliminary animal studies using the uremic rat.

Proposed Course: This contract is being terminated.

Contractor: University of Naples

Amount: $15,750

Title: Studies on Amino Acids in Uremia

Objectives:

To study selected facets of amino acid metabolism in chronic uremia with emphasis on phenylalanine, tryosine and histidine. To establish the true essential amino acid requirements in chronic uremia [which differ from those of normal man]. To investigate the feasibility of special diets for uremic patients based on keto acid analogues of the amino acids essential for maintenance in uremia.

Major Findings:

The principal investigator has demonstrated that in chronic uremia the ordinarily nonessential amino acid histidine is indeed essential. Histidine supplementation improves uremic anemia and nitrogen balance. Preliminary studies have indicated that uremic patients can utilize the keto acid analogues of essential amino acids in endogenous protein synthesis. It is of particular interest to note the specific action of histidine in improving hematocrit of uremic patients. This is consistent with a similar observation in patients with rheumatoid arthritis of long standing who received histidine supplementation for a completely different reason; the histidine did not improve the rheumatoid arthritis, but invariably significantly improved their low hematocrits [this was a three-clinic study in the eastern United States].

Proposed Course:

With the aid of dietary and metabolic studies and other relevant experimental investigations in uremic patients the contractor will establish the true essential amino acid requirements in chronic uremia [which differ from those of normal man]. Subsequently, the contractor will investigate the potential usefulness of diets of special composition containing all of the amino acids which are found to be essential in uremia, for improvement of hemoglobin status and nitrogen balance of uremic patients.

The contractor will investigate the possible utilization by uremic subjects of keto acid analogues of essential amino acids in endogenous protein synthesis. The contractor will also investigate problems of nitrogen storage under normal conditions and in uremia by tracing free and conjugated amino acids in various tissue compartments.

Contractor: University of Naples

Amount: $30,000

Title: Studies on Oxystarch in the Treatment of Uremia

Objectives:

To develop a chemical compound which can be administered per os to sequester urea and possibly other uremic toxicants in the gastrointestinal tract, and to develop with the aid of such a compound a suitable adjunctive treatment for patients in chronic renal failure. Also, to explore the feasibility of using oxystarch as a sorbent of uremic wastes in artificial kidney dialysate solutions.

Major Findings:

A chemically well defined polyaldehyde has been obtained from controlled periodate oxidation of starch. The material does not contain iodine residues and has been proven nontoxic in long-term mouse feeding experiments. The material is stable and non-dialyzable. One hundred grams of "oxystarch" (OXS) bind 33 grams of urea. When given by mouth to uremic patients in divided doses, 20 grams of oxystarch per day result in a daily reduction of 20 milligram percent of blood urea nitrogen. Fecal nitrogen content increases correspondingly and the increased fecal nitrogen can be ascribed to large quantities of urea-derived ammonia irreversibly trapped by oxystarch during passage of the gut. Oxystarch is not hydrolyzed in the intestinal tract, nor absorbed.

The latest experiments have demonstrated that OXS does not chelate food-nitrogen during its passage through the gastrointestinal tract. Controlled experiments in patients receiving OXS who are either fed a Giordano-Giovannetti diet or a regular hospital diet (which contains a higher level of protein) or a protein-free diet have shown that the level of protein intake with the food does not influence the amount of urea/ammonia binding by oxystarch upon passage of the gastrointestinal tract in uremic patients.

Recent experiments have also shown that oxystarch, despite the fact that it is soluble in the human gut, is soluble in dialysate solutions, thus it cannot be used as a urea-specific binder in the dialysate of artificial kidneys.

Proposed Course:

The investigator will test the use of OXS in controlled larger clinical trials for prolonged periods of time in accordance with carefully outlined plans. Also, the contractor will prepare oxystarch in quantities large enough for clinical and laboratory experimentation in the United States as specified by the Artificial Kidney - Chronic Uremia Program which will distribute this material to American investigators for specific, sponsored studies.

A number of other "oxystarches" have been manufactured by commercial sources in the United States and abroad. They, however, have not been successful in urea/ammonia binding in the gut of uremia patients -- or only partially so. The investigator will therefore also make an effort to chemically and physically characterize "OXS/Naples" in comparison with "OXS/Miles" and "OXS/Sumner" [the latter two have shown definite differences in physical and chemical properties as compared with "OXS/Naples."]

Contractor: National Institute of Scientific Research

Amount: $77,453

Title: Development of Modified Polycarbonate Membranes for Hemodialysis

Objectives:

The objective of this program is to develop polycarbonate hemodialysis membranes with mechanical and transport properties superior to those of Cuprophan. Markedly enhanced permeability to "middle molecules" (300-5000 molecular weight), with controlled ultrafiltration rates are particularly desired.

Major Findings:

Emphasis has been placed on scale-up synthesis and development of casting procedures for membrane for clinical evaluation in Kiil-type dialyzers. Polymer synthesis scale-up to one pound/run was achieved and transition from small-scale hand casting of membrane (6-in. width) to developmental casting on production-type machinery (15-in. width) was also achieved. An advance has been the development of a water gelation process for membrane formation. This step required reformulation of the casting solution using water-miscible solvents. Earlier polycarbonate membranes were cast from chloroform solution and gelled in alcohol, a procedure which was considered impractical on a large scale. The new machine-cast water gelled membranes are tougher than the alcohol-gelled membranes, while retaining the same outstanding transport properties.

Transport data shows that a membrane with a controlled ultrafiltration rate (1.58 x Cuprophan) and comparable clearance of low molecular weight species, can have considerably enhanced "middle molecule" transport. Recent results with newly prepared membranes indicates that U.F. rates can be lowed further (1-1.5 x Cuprophan) with only minor effects on other key properties.

Preliminary _in_ _vitro_ dialyzer evaluation has been started with the polycarbonate membrane.

Proposed Course:

This program is being continued to develop a reliable process for the preparation of polycarbonate membrane, to solve storage stability problems and to furnish membrane for preliminary clinical tests.

Contractor: New York Medical College

Amount: $119,100

Title: Toxins in Uremia

Objectives: The objective of this project was evaluation of a special protein restricted diet for the alleviation of the subjective and objective signs and symptoms of uremia and an attempt to use this diet to elucidate the toxic factors in uremia.

Major Findings: In the first four years of this contract the New York Medical College group treated 70 patients with a modified Giovannetti-Giordano diet. In patients with GFRs of greater than 4ml/min, dietary therapy was usually adequate in reducing gastrointestinal symptoms and increasing the patients' comfort although it in no way slowed the progress of the underlying renal disease or its complications. Studies were also undertaken to prolong the interval between dialyses in patients with GFRs of less than 4ml/min. Diet-dialysis therapy was always associated with the eventual development or worsening of motor neuropathy which reauired intensive dialysis for reversal.

The results of these investigators indicate that a substance or substances inhibiting the activity of the enzyme transketolase, for which thiamine pyrophosphate is the coenzyme, is found in the plasma of chronic uremia patients exhibiting signs of peripheral neuropathy. This inhibitor is not significantly diminished by diet therapy, but can be removed by hemodialysis. The enzyme affected occupies a central position in the pentose phosphate pathway of non-oxidative glycolysis. The demonstration of an inhibitor of erythrocyte and brain transketolase in plasma from uremic patients suggests a possible mechanism for the development of uremic neuropathy, and its response to hemodialysis. The contractor feels that this enzyme model system may offer a technique for investigating the metabolic lesions of uremia and may serve as an objective measure of response to dialytic therapy.

Proposed Course: Termination.

Contractor: University of North Carolina

Amount: $89,000

Title: Antithrombogenic Surfaces: Platelet-Interface Reactions

Objectives: Study the composition of the layer of plasmatic components adsorbed to materials exposed to blood, and to characterize this layer as to its effect on blood platelets.

Major Findings: These investigators indicate that there are morphological differences between the adsorbed layers formed on certain polymeric and nonpolymeric test materials and that an adsorbed layer is universally present between adherent platelets and the surface test material. It appears that it is this adsorbed layer and not the surface of the test materials which determines thrombogenicity. The chemical nature of the test material may control the composition of the adsorbed layer and through this, its thrombogenicity, or it may merely control the rate of deposition of the adsorbed layer.

Proposed Course: Continue characterization of the blood-polymer layer and its role in material thrombogenicity. He is also going to evaluate various polymers in an *in vivo* situation by placing vena cava rings in Rhesus monkeys.

Contractor: North Star Research and Development Institute

Amount: $109,620

Title: Development of a New Concept in Membrane Structure for Application in Hemodialysis

Objectives:

The objective of the contractor has been to develop ultrathin (5,000-20,000 angstroms in thickness) membranes for hemodialysis.

Major Findings:

A new ultrathin membrane has been developed based on cellulose acetate and showed increases in clearance over those of Cuprophane PT-150 from 30 percent for low molecular weight metabolites to 90 percent for middle molecular weight metabolites. A laboratory pilot production facility was designed, assembled and put into operation for continuous casting of double layer ultrathin membranes. Five hundred to 750-foot lengths of supported bilayer films have been successfully turned out and routine production of 1000-foot rolls seems easily attainable. A clinical evaluation is now being performed after encouraging animal results were achieved.

Proposed Course:

Completion of *in vivo* acute and chronic clinical testing of the membrane and support composite.

Contractor: University of Oregon

Amount: $21,000

Title: Platelet Function Studies in Chronic Dialysis Patients

Objectives: This new contractor will study platelet functions in uremic dialyzed and non-dialyzed patients. In addition, stabilized dialyzed uremics will have their dialysis regime altered to one of infrequent dialysis and platelet function studies will be conducted in these people. The investigators hope to demonstrate that platelet function studies are a measure of adequate dialysis.

Contractor: University of Pennsylvania

Amount: $189,451

Title: Blood Purification by Ultrafiltration and Reconstitution

Objectives:

To study the factors involved and develop a system for cleansing the blood by ultrafiltration and replacing the ultrafiltrate with sterile reconstituting solution containing appropriate concentrations of normal body constituents removed in the ultrafiltrate.

Major Findings:

An improved membrane and potting procedure have been developed for the hemodiafiltration unit. Sterility and pyrogen control have been achieved in the production of the units. Clearance of "middle molecules" e.g., inulin with a molecular weight of 5200, has proven to be comparable to that of the human kidney (100 ml/min.) Clinical application of diafiltration for six hours on several occasions has not been associated with any untoward signs or symptoms.

Proposed Course:

Continue toward present objectives with acute and chronic clinical trials as well as improved fabrication techniques of hemodialysis units.

Contractor: University of Pisa

Amount: $25,185

Title: Albumin, Urea and Insulin Metabolism in Chronic Uremia

Objectives:
To determine the turnover and biochemical aberrations of albumin, urea and insulin metabolism in uremia.

Major Findings:
Sophisticated determination of albumin catabolism through a new triple tracer technique has shown that the intravascular albumin mass is normal in patients on the low, selected protein (Giordano-Giovannetti) diet (even in patients maintained on it up to five years). The extracellular albumin mass, total albumin mass and extracellular to intravascular ratio were reduced in both short-term and long-term patients. The total intravascular albumin mass was preserved within a normal or near normal range. Albumin metabolism was normal in controls and long- or short-term patients. The diet is not directly responsible for the observed albumin depletion since albumin synthesis continues at a normal rate in all cases. Apparently toxic factors of the uremic state itself are responsible.

With regard to insulin it was found that catabolism of insulin was slowed down considerably in uremic patients -- apparently because normally functioning renal tissue is required for a normal rapid breakdown of insulin in the body. It was also found, however, that the rate of secretion of insulin from the pancreas is reduced in uremia -- apparently because of an as yet unknown toxic suppression of normal insulin synthesis in the pancreas or secretion from the beta cells.

Proposed Course:
Continue to evaluate albumin metabolism in patients who are uremic and on a low protein diet, investigate the urea metabolism in these patients, and study albumin, insulin and growth hormone turnover and distribution in uremic patients who are on or off hemodialytic therapy.

Contractor: Research Triangle Institute

Amount: $00.00

Title: Improved Non-thrombogenic Materials

Objectives:

Goals are to prepare a number of potential non-thrombogenic surfaces by grafting hydrophilic polymer chains to other polymeric substrates, and to test such surfaces for blood compatibility in vitro and in vivo.

Major Findings:

The substate materials have been chosen to demonstrate the general applicability of this method to polymers of varied chemical composition and mechanical properties. Polyethylene, Polydimethylsiloxane (Silastic), polyurethane and polyvinyl chloride have been successfully surface grafted in the form of tubing of various dimensions.

The surface grafting of hydrophillic monomers to the various substrates has been accomplished by two techniques. The first is pre-irradiation of the polymer in oxygen to form surface peroxides which are subsequently thermally decomposed in a monomer solution to initiate surface grafting. The second is the mutual irradiation of the polymer in a monomer solution. Both of these methods have been used successfully with each of the polymeric materials; the choice of method being determined by the monomer.

The Thrombogenicity of the materials has been evaluated in vitro by exposing the grafted surface of the tubes to fresh whole human blood for thirty minutes than measuring the partial thromboplastin time (PTT) and the Thrombin time (TT) of the exposed blood. Polyethylene and siliconized glass or Silastic were used as control surfaces of increasingly severe comparison. The criteria of nonthrombogenicity is the zero time PTT. This is the PTT measured on blood drawn directly into a syringe containing sodium citrate solution thus interrupting the coagulation sequence before the blood is exposed to any foreign surface. Three different surface grafts to Silastic (three different hydrophillic, non ionic monomers) had PTT values greater than the zero time value and were therefore carried into the in vivo system.

The in vivo test is a modification of the Gott ring test in which grafted tubes 32mm in length with an I.D. of 3mm are implanted in the inferior vena cava of dogs. The flow rate of the blood is measured at the proximal end of the prosthesis using an electromagnetic flow probe. Venous pressure and arterial pressure are monitored during the test period. Platelet counts, blood gases, hematocrit, PTT, assays for factors V and VIII are also measured throughout the duration of the test.

Proposed Course:

This program has been discontinued.

Contractor: Research Triangle Institute

Amount: $157,000

Title: National Dialysis Registry

Objectives: To develop a national registry of all patients on hemodialysis.

Major Findings: The National Dialysis Registry collects data from virtually all centers engaged in dialysis in the United States, and prepares from the data statistical analyses for use by the NIAMDD and quarterly reports to all cooperating centers. The data include numbers of patients in center or home dialysis, changes in status of these patients, cause of death in patients that expire, length of time on dialysis, etc. Confidentiality of the center and patient identities is maintained. Analyses of death by primary cause and multiple decrement life tables are also prepared.

The establishment of the Registry, although primarily devoted to vital-statistics fact finding, will allow, should more in-depth be required, the broadest possible base for statistically valid sampling which would not put a large load of data collection on any one center.

Contact is also maintained with the European Transplant and Dialysis Registry, the Organ Transplant Registry, and the Canadian Dialysis and Transplant Registry.

Proposed Course: To continue obtaining, cataloging, storing, and making available data of patients on dialysis.

Contractor: St. Francis Hospital, University of Hawaii

Amount: $20,000

Title: Effect of reuse of Cuprophan and Cellophane Coils on (a) Ultrafiltration and (b) Clearance of Urea, Creatinine, Uric Acid, Phosphate, Sulfromophthalein, Vitamin B_{12} and Inulin.

Objective:

This new contractor will study the effects of various storage techniques on dialysis membranes in an attempt to draw a conclusion as regards the relative efficacy of different storage techniques.

Contractor: Stanford Research Institute

Amount: $87,400

Title: Amino Acids, Peptides and Proteoses in Uremia in Man

Objectives: To investigate the amino acid, peptide and proteose composition of uremic plasma, urine, and dialysate and relate it to that found in normal individuals.

Major Findings:

Studies on the Intestinal Absorption of Tryptophan. Their earlier studies of the intestinal absorption of tryptophan in normal subjects and patients have been expanded; they compared results in 10 normal subjects with those in 8 end-stage uremic patients and with those in 6 patients maintained by hemodialysis. At all times, ranging from 30 to 210 minutes following an oral test dose of 25 mg tryptophan/kg, they found that tryptophan levels in patients were about one-half the levels found in normal subjects. Levels in the two groups of patients were not markedly different, showing that maintenance dialysis does not correct the metabolic defect in end-stage uremia that causes the lower levels of tryptophan. In most of the individuals (8 normal subjects and 9 patients), tracer quantities of the nonmetabolized marker amino acid, ^{14}C-cycloleucine, were given simultaneously with the tryptophan dose. All subjects and patients exhibited essentially the same pattern of absorption of the ^{14}C-cycloleucine, showing that no absorption defect for cycloleucine occurs. An assessment of the rate of disappearance values for tryptophan of 1.9 (normal), 1.6 (end-state uremics), and 1.3 hours (dialysis patients). These values suggest slight-to-moderate acceleration of metabolism of tryptophan in the patient groups. The larger differences in rate of removal of tryptophan suggests that other abnormalities, such as malabsorption, are also present.

Studies on the Binding of Tryptophan by Plasma Proteins. A significant physiologic difference between tryptophan and all other amino acids in plasma is that only tryptophan is bound to plasma proteins. Earlier work by others established that albumin is the protein that binds tryptophan. The investigators earlier work used procedures that deliberately disassociated the tryptophan-albumin complex during deproteinization before analysis and, therefore, had measured total tryptophan—i.e., the sum of protein-bound and unbound tryptophan.

To determine if patients undergoing dialysis differed from normal subjects in the extent of binding of tryptophan by plasma proteins, they employed Amicon membrane filtration cones to obtain ultrafiltrates of plasma from 12 normal subjects and 10 uremic patients/

The extent of binding was calculated from the tryptophan found before and after ultrafiltration. Albumin concentrations of the plasma were determined concurrently be electrophoresis on cellulose acetate strips. Both total plasma tryptophan and plasma albumin were significantly lower in the patients than in the normal group. The extent of binding of tryptophan in the normal group averaged 80 (± 1) percent, a value similar to that found in normal subjects by others. The mean percentage of tryptophan bound--44 (± 5) percent--in the patients was significantly lower. In both groups, the extent of binding of tryptophan and the level of plasma albumin were positively correlated. Further examination of the interrelationship among bound and unbound tryptophan and albumin levels was made by plotting the ratio of bound (B) to unbound (UB) tryptophan versus albumin. Both the normal and patient groups showed positive correlations of B/UB tryptophan versus albumin with no overlap between the two groups; but the slopes of the regression lines for the two groups were not different and formed one continuum. This suggests the possibility that not only does tryptophan influence albumin synthesis but also that the lower extent of binding of tryptophan in the patient group may result in accelerated degradation of albumin. It has been reported that tryptophan exerts a protective effect against the proteolysis of albumin when it is complexed with the albumin.

Proposed Course:

To determine whether the lower binding of tryptophan in uremic patients is due to an abnormal albumin or to the presence of competing solutes for the tryptophan binding site on albumin.

Contractor: Southern Research Institute

Amount: $37.943

Title: Activated Carbon Fibers for Use in Artificial Kidney Devices

Objectives:

1. Explore the utility of activated carbon fibers for use in artificial kidney devices.
2. Serve as a source of activated carbon fibers for the use of other investigators.

Major Findings:

1. A partially suitable fiber can be produced in coil form by carbonization from Saran.
2. A particularly interesting and strong fiber exists in loaded monofilaments of activated carbon (LOMAC). The LOMAC material can be produced in the hollow fiber format.

Proposed Course:

Prototypes of activated carbon hemoperfusion devices will be constructed and submitted to another investigator for *in vivo* testing in animals.

Contractor: Tecna Corporation

Amount: $31,940

Title: Clinical Evaluation of Microporous Tissue Interface Dialysis Shunts and Catheters

Objectives:

Develop microporous tissue interface dialysis shunts and catheters for clinical use in hemodialysis and peritoneal dialysis.

Major Findings:

The techniques for fabricating a shunt consisting of three elements (the case polyurethane tubular structure, the external microporous polyurethane tissue fixation coating, and the internal polyurethane coating of low thrombogenicity) have been developed. Methods of quality control of materials, blood compatibility testing, sterilization and packaging have been established. Acute clinical trials of hemodialysis shunts have begun.

Proposed Course:

Completion of *in vivo* clinical trials of tissue fixation A-V shunts and peritoneal dialysis catheters.

Contractor: University of Texas at Austin

Amount: $19,000

Title: Analysis of Transport Parameters in Hemo- and Peritoneal Dialysis

Objectives:

This new project will employ compartmental analysis to study transport resistances between body pools as a function of molecular size or weight. Similar information will be obtained for the peritoneal dialysis situation.

Contractor: Tulane University

Amount: $100,000

Title: Studies on the Mechanism of Anemia in Patients with Renal Disease

Major Objectives:

The contractors are attempting to examine hematologic parameters in patients with varying degrees of renal function impairment. Particular emphasis is being placed on the study of erythropoietin, and on renal erythropoietic factor.

Major Findings:

Thirty-five patients have entered this study. Preliminary results indicate that the anemia in these uremic patients consists of a significant degree of marrow depression. A few patients have displayed elevated ESF levels.

Proposed Course:

To continue the study of anemia as it develops over the range of renal excretory function in selected patients with renal disease. To study the acute and chronic effects of hemodialysis on circulating red cells and erythroid function. To determine the erhthropoietic response of anemic uremic marrow to erythropoietin and to androgens. To determine the plasma levels of erythropoietin in patients with the anemia of chronic renal disease using a sensitive radioimmunoassay for ESF.

Contractor: University of Utah

Amount: $38,800

Title: A Development of Single Needle Dialysis

Objectives:

Objective of this contract is to develop a device whereby a patient can be dialyzed with the use of a single needle puncture into the arteriovenous fistula rather than the currently common practice of using two needles for dialysis.

Major Findings:

The single needle technique has become a clinical reality, thus far being used successfully on 33 patients. The method has been safe and comparison of patients first treated with two needles, then with single needle, showed little difference. There have been no accidents.

Proposed Course:

Continue present objectives and further define clearances, recirculation and hemolysis with *in vivo* and *in vitro* studies.

Contractor: University of Utah

Amount: $60,163

Title: Fully Automatic Peritoneal Lavage System Using Reverse Osmosis

Objectives:

This is a contract to develop a safe fully-automatic peritoneal dialysis system using reverse osmosis as the source of water; to determine the relative advantages of peritoneal dialysis on patients who for various reasons may not be suited to maintenance hemodialysis; to test whether pre-pubertal or very small children can be maintained by peritoneal dialysis for prolonged periods and whether daily dialysis of this nature will improve nutritional status, growth, and sexual maturation.

Major Findings:

Numerous technical difficulties were encountered in the design and construction of the fully automatic peritoneal lavage system, especially with the reverse osmosis unit. However, the truly innovative features of the system, the outflow chamber and the counting unit, have functioned very well. A new reverse osmosis unit will be purchased from another source and this should solve a great number of problems.

Proposed Course:

The contract has not been extended for another year. However, the principal investigator will continue research at his own expense with an eye towards development of a clinically useful apparatus.

Contractor: University of Utah

Amount: $55,606

Title: Adsorbent Hemoperfusion

Objectives:

To investigate and develop the concept of directly contacting blood on activated carbon for the purpose of removing uremic "toxins".

Major Finding:

In vitro adsorption isotherms have been generated for creatinine, salicylic acid and nembutol. Coating the carbon with glutaraldehyde cross-linked albumin or with polyhydroxyethyl-methacrylate (Hydron) does not significantly reduce adsorption rates or capacities.

In vivo experiments with sheep have shown dramatic creatinine and uric acid clearances through a 10 gram cartridge. Poly-HEMA coated carbon exhibits less platelet depletion than uncoated controls.

Proposed Course:

Charcoal embolization will be investigated in detail by means of autopsy and histopathological studies on acutely treated sheep. Formed element balances will be done and the in vitro adsorption studies will be expanded. Chronic experiments will be performed and the possibility of immunologic actions of albumin coatings will be studied.

Contractor: University of Utah

Amount: $40,000

Title: Thyroid Function in Patients in Chronic Renal Failure

Objectives:

The investigators are to study the thyroid function in patients with chronic renal failure by evaluating and defining the abnormalities in thyroid dysfunction in uremic patients and patients on hemodialysis.

Contractor: Vanderbilt University

Amount: $40,000

Title: Neurobehavioral and Clinical Responses to Hemodialysis, with Reference to Large Surface Area Dialyzers

Objective: The investigator is attempting to document neurobehavioral alterations in the uremic state.

Major Findings: To date the investigator has demonstrated that EEG power spectrum analysis and certain cognitive functions deteriorate in the patient prior to clinical observations that would lead a nephrologist to believe that a patient should be dialyzed.

Proposed Course: The investigators will continue to pursue their objectives employing a variety of new tests.

Contractor: The University of Washington

Amount: $135,873

Title: Cannula Research for Hemodialysis

Objectives:

Investigate by means of a broad approach improvement of an access to the circulation.

Major Findings:

The sheep has been established as a model for cannula testing. A completely externalized vessel for easier insertion of dialysis needles was successfully created in sheep but patient acceptance of such an idea was determined to be very low. Functioning artificial guts have been maintained in several sheep and in one human patient for several months. Coumarin therapy was found to definitely prolong cannula life in sheep. Preliminary studies on the promising fistula catheter were begun.

Proposed Course:

Continue the current objectives utilizing the animal shunt model, with particular emphasis on development of the fistula catheter.

Contractor: University of Washington

Amount: $175,000

Title: Clinical and Engineering Research on Hemodialysis Procedure

Objectives:

To explore and to improve upon clinical dialysis strategies designed to demonstrate that toxic middle molecules cause uremic nephropathy (as measured by motor nerve conduction velocity (MCNV) by:

1. optimization of dialyzer membrane combination and clinical protocols;
2. study of effect of residual renal function upon dialysis;
3. verification of middle molecule hypothesis by use of: a) small area dialysis, b) reduced time dialysis; and
4. relationship of dialysis to a deficiency syndrome.

Major Findings:

1. Data from in vitro studies using UF-100 twin coil and Dow HFAK Model 3 indicate poor clearance of molecules with an MW > 2000, as compared to clearance with a standard Kiil. Patients dialyzed with either of the above units did not develop peripheral neuropathy.

2. A patient who had a GFR= 0.22 ml/min did not develop neuropathy on a dialysis regimen which would have been predicted to allow middle molecules to accumulate.

3. Small surface dialysis in a patient dialyzed 27 hrs/week for 6 months has been performed and no significant change in MNCV has been observed.

Proposed Course:

Study of:

1. Optimization of dialyzer membrane combinations and clinical protocols a) mathematical analysis of contribution of ultrafiltration to middle molecule removal; b) experimental verification of the theoretical calculations; c) correlation of dialyzer protocols with solute MW; d) in vivo evaluation of new membranes; and e) clinical evaluation of variations in dialysis times and frequency.
2. Effect of residual renal function (RRF) with: a) mathematical models; b) clinical study of effect of RRF on hemodialysis; c) evaluation of techniques for measurement of minute levels of GFR; and d) correlation between urine volume and GFR.
3. Continuation of work on small area dialysis to correlate with MNCV.
4. Dialysis techniques that can induce deficiency syndromes will be used and evaluated.

Contractor: University of Washington

Amount: $86,320

Title: Development of a Low Cost Home Peritoneal Dialysis System

Objectives:

Continuing development and evaluation of automated, home peritoneal dialysate supply and delivery systems.

Major Finding:

A system employing an on line reverse osmosis water supply has been evolved and is now undergoing clinical trial.

Proposed Course:

Continuing clinical and technical evaluations as well as minor equipment modifications will be pursued, and information which will provide a critical comparison between hemo- and peritoneal dialysis will be sought.

Contractor: University of Washington

Amount: $50,000

Title: Acid-Base Chemistry and Human Bone

Objectives: The contractor will:

(1) Study the buffering capacity of bone by evaluation of the lobile carbonate fraction in uremic, acidotic patients.

(2) Classify bone biopsies on a histologic basis and relate biopsy diagnosis and changes to primary disease process and therapy.

Major Findings: The investigator has biochemically characterized normal bone, normal bone maturation, and bone in several disease states.

Proposed Course: The major effort now is to determine how various therapy modifies diseased bone histology and chemistry.

Contractor: University of Washington

Amount: $81,000

Title: Pharmacokinetic Studies in Chronic Renal Disease and Hemodialysis

Objective: The investigators are attempting to characterize a number of drugs in the uremic state - specifically they want to determine mode and rate of excretion, volumes of distribution, mechanisms of metabolism, etc. In order to do this they are employing both standard chemical measurements for some drugs [where these have been established] and in other cases, developing their own.

Major Findings: These investigators have developed data on the pharmacokinetic characteristics of lasex and gentamicin in patients with acute renal failure and in chronic uremics being dialyzed and not being dialyzed.

Proposed Course: These investigators will continue to develop data to the point of statistical significance in the drugs mentioned above and in addition will branch out to other drugs including procainamide, kanamycin, streptomycin, and ethambutol.

Contractor: University of Washington

Amount: $78,728

Title: The Anemia of Renal Failure

Objectives:

To study aspects of the anemia of chronic renal failure in a suitable animal model by characterizing:

1) Oxygen transport mechanisms in the normal and anephric state.
2) The relationship of erythropoietin and marrow production to changes in oxygen delivery in the normal and anephric state.
3) The effect of chronic hemodialysis on erythropoietic function and oxygen transport in the anephric state.
4) The role of inhibition of marrow function in the uremic state.

Major Findings:

During the past year the investigators have succeeded in establishing an uremic, anemic sheep model that is stable and that may or may not require bi-weekly hemodialysis. The model is created by infarcting all but a small part of one kidney and removing the opposite kidney. Preliminary studies of the anemia of renal failure and oxygen transport reveal that erythropoiesis and erythropoietin production in the intact sheep is similar to normal man and red cell production in the uremic sheep is markedly decreased, similar to the hypoproliferative anemia of chronic renal failure in man. However, the sheep does not have a compensatory oxygen transport mechanism similar to man when anemia develops, since there are no significant increases in intraerythrocytic levels of 2, 3-DPC or a rightward shift in the oxyhemoglobin dissociation curve. Enough physiologic studies have been performed in the intact and uremic sheep that the goals of the original contract request can be eventually achieved.

Proposed Course:

This contract is being terminated.

Contractor: University of Wisconsin

Amount: $00.00

Title: Prepartion of Vitamin D Metabolites and Analogs

Objectives:

The objective of this contract is to synthesize 1α, 25-dihydroxycholecalciferol and other vitamin D analogs or metabolites for clinical evaluation in uremic patients.

Major Findings:

The principal objective has been the preparation of larger amounts of the natural metabolite $1\alpha,25\text{-}(OH)_2D_3$ (I). Since the original synthesis of metabolite I involved a fairly lengthy synthetic route, some effort has been devoted to the development of a shorter procedure for the preparation of the requisite 1α, 3β-dihyroxy-Δ^5-steroid system. A short and convenient route to 1-hydroxylated steroids has now been developed. The new scheme has been explored with cholesterol as a starting material, which serves both as a convenient model and as a starting point for the eventual synthesis of the active vitamin D analog, 1α-OH-D_3. The synthesis is, however, equally applicable to the preparation of $1\alpha,25\text{-}(OH)_2D_3$ as well as other 1,3-dihydroxyvitamin analogs. Using this procedure the contractor has prepared ca. 500 mg of a dihyroxy precursor which currently is being converted to the active vitamin, 1α-OH-D_3.

The contractor has started to apply this new procedure to the synthesis of $1\alpha,25\text{-}(OH)_2D_3$. On a small scale the required intermediates, a trienone and an epoxide have been prepared. Enough starting material is available to prepare ca. 20-50 mg of the metabolite within the next two months.

Proposed Course:

It is anticipated that within the two year period of this contract the major objectives will be successfully completed.

Contractor: Nutrition Program, HSMHA (Interagency Agreement)

Amount: $6,560

Contractor: American Institute of Nutrition (NIH 70-2086)

Amount: $39,118

Title: Food Composition Table for use in East Asia (U.S.-Japan Program)

Objectives:

The primary objective of this project is the preparation of a table of food compositions for foods used in the East Asian countries (Burma, Thailand, Laos, Vietnam, Cambodia, Malaysia, Indonesia, Philippines, Taiwan, Korea, and Japan). This includes indigenous food products as well as foodstuffs imported to these countries which are consumed locally. The first Far Eastern Food Table was issued in 1945 under the auspices of the Committee on International Food Value Problems of the National Research Council, U.S.A. Although it was revised and enlarged in 1952 as Agriculture Handbook No. 35, "Composition of Foods Used in Far Eastern Countries," it is not applicable to conditions in 1969. Comprehensive food tables for local foods in East Asia are essential for and basic to any attempt of improving the nutritional state of East Asian populations through a combination of nutrition education or nutrition improvement through introduction of innovations in the local food consumption.

Major Findings:

The Principal Investigator, Dr. Leung, who was responsible for the preparation of the first Far Eastern Food Table immediately after World War II, has collected all available food data through correspondence and through personal visits in the various countries. In addition, there is active collaboration with the Nutrition Division of the Food and Agriculture Organization of the United Nations, through Dr. Autret and his staff who have accumulated a considerable body of data at FAO Headquarters in Rome and are constantly generating new information in regional FAO offices in Asia.

The information collected includes: data on moisture, "proximate composition," minerals, vitamins, amino acids, fatty acids, trace elements, refuse, and local methods of preparation, processing and preservation of foods, etc. Whenever feasible, local names used in various Far Eastern countries with the corresponding English and scientific names are recorded and collected. The result of this Project will be printed as a joint publication "Food Composition Table for Use in East Asia" -- NIAMDD will be responsible for the printing of the English version, and FAO will undertake the translation and printing in French. The format of the food table will follow closely the one previously designed for Africa.

Data collection was begun in the winter of 1969-70 and has progressed very satisfactorily. These have been evaluated and collated into tentative food tables which were "frozen" into a final format in the winter of 1972.

Proposed Course:

During the months of January and February 1973 camera-ready copy was prepared for the two major books which will result from this project: (1) "Food Composition Tables for Use in East Asia," and (2) "Bibliography of Food Composition Values," which gives the origin of the food values in these tables. This material is about to be sent to the Government Printing Office for final printing. The project officer and FAO headquarters in Rome will negotiate a modus operandi of the distribution of the resulting publications during a meeting of the FAO staff some time in the early Fall of 1973. It is contemplated that the material be distributed as widely as possible among nutritionists in the United States with emphasis on those active in the international nutrition sphere. FAO, in turn, will distribute this material broadly in the East Asian countries both in the original English version as well as in the French translation (Indo-China).

Contractor: International Rice Research Institute (PH-43-67-726)...

U.S.-Japan Program

Amount: $40,165

Title: Improvement of the Protein Content of Rice

Objectives:

The primary objective of this project is to determine the feasibility of incorporating a high level protein into commercially produced rice in Southeast Asia. Presently used commercial varieties of rice contain from seven to seven and one-half percent protein. The biological quality of rice protein is fairly high and an increase in the protein level in rice would go far toward alleviating protein undernutrition in countries for whom rice is the mainstay of subsistence. Since 60 percent of the world's population subsists primarily on rice, the implications for an amelioration of the chronic protein shortage in many underdeveloped countries, should this project prove successful, are quite obvious. If the present protein level of rice could be elevated by several percentage points an automatic and sweeping nutritional improvement would take place in major areas throughout the world.

Methods Employed:

The modus operandi involves the screening for high protein content, of over 7,000 varieties of rice archived in the International Rice Research Institute collection, followed by selective breeding and development of a commercially feasible variety which incorporates an improved protein level together with all the necessary attributes which would make this rice attractive to the average Southeast Asian rice grower, namely high yield per acre, resistance to endemic diseases, strong response to fertilization, and -- most important -- desirable organoleptic qualities with respect to stickiness, mouth-feel and taste.

Major Findings:

Over 100 rice varieties have been identified among the many thousands tested which contain between 13-1/2 to 17-1/2 percent protein. Thus far six high protein strains of rice (some from Hungary and some from Southeast Asia) have been successfully crossbred with a new rice variety (IR8) which has been developed previously at the International Rice Research Institute and which possesses near-ideal plant characteristics including disease resistance and a very high yield per acre. Some of the new hybrid lines have shown as high a protein content as the high-protein parent. Others, of fairly acceptable "plant type" have a protein content of +-11 percent, i.e., contain 50 percent more protein than currently produced commercial rice. Based on the most recent breeding work involving progeny of successful crosses between the six high-protein strains and IR8 the project staff has shown that the protein content of rice is indeed a genetically determined characteristic. Protein efficiency ratio (PER) studies, using rats, of several parent rice strains have been carried out by Dr. Bressani at the Institute of Nutrition of Central America and Panama (INCAP) and future PER studies of the new hybrid varieties are contemplated. Dr. Helen Clark of Purdue University has demonstrated in nitrogen balance studies involving students that the experimental high-protein rice which contained 1.8 times as much protein as Bluebonnet rice (a commercially controlled rice containing the conventional 7 percent protein) caused significantly higher nitrogen retention ($P < 0.01$), which is attributable to the increased amounts of essential amino acids. The respective mean positive balances were 1.41 = 0.89g and 0.24 = 0.31g. Addition of supplementary nitrogen to make the Bluebonnet rice isonitrogenous with the BPI-76-1 variety did not cause a statistically significant improvement. All subjects were in negative balance when 320g Bluebonnet rice were made isonitrogenous with 480g of the same rice by adding nonspecific nitrogen.

During the last year new feeding studies have started in infants in Manila under the supervision of Dr. Intengan in order to demonstrate the utility of the high-protein rice strains not only in improving nitrogen balance in adults, but in supporting active growth in infants.

Significance:

(Please see above under Objectives.) Ordinary commercial rice does not contain enough protein to support normal growth and development of infants and children maintained on a predominant rice diet, and does not protect the adult population from serious protein under-nutrition. Rice is the principal food of 60 percent of mankind. If the present protein level of rice (approximately 7-1/2 percent) could be elevated by several percentage points an automatic and sweeping nutritional improvement would take place in all the populations for whom this one crop represents the nutritional mainstay. Such a development would concomitantly relieve the current burden of the United States which supplies hundreds of millions of dollars' worth of supplementary foods to underdeveloped rice-eating countries. The potential nutritional improvement among Southeast Asian populations and underdeveloped rice-eating countries throughout the world is an important aim of the U.S.-Japan Program and of the applied nutrition program of the Institute.

Proposed Course:

The high-protein rice lines isolated thus far have displayed, during the last year, a certain susceptibility to leaf spot and leaf blight. Therefore crossbreeding experiments will continue to breed into the new strains resistance to these two plant diseases. In the interim, side by side with these crossbreeding efforts which are contemplated to require one year (two seasons), further nutrition studies in infants and adults will be carried out in cooperation with Dr. Intengan in Manila and Dr. Scrimshaw in Boston.

Eventually, because of a change in the guidelines of the U.S.-Japan Program, the agricultural portion (breeding experiments) of this project will have to be funded by the International Rice Research Institute or some other source; the human nutrition aspects of this very promising project will continue to be pursued by NIAMDD.

THE ANNUAL REPORT SUMMARY
EPIDEMIOLOGY AND FIELD STUDIES BRANCH

SOUTHWESTERN FIELD STUDIES SECTION

Dr. Arthur Dorf received the 1972 J.D. Lane Award of the Commissioned Officers Association and Clinical Society of the United States Public Health Service, prior to completion of his appointment as Staff Associate with the Section. Dr. Dorf received the award for a paper entitled RETINOPATHY IN THE PIMA INDIANS.

Dr. Stephen E. Dippe joined the staff of the Section in October 1972 as Associate Chief. Dr. Dippe's background in both endocrinology and epidemiology will strengthen the Section considerably.

Dr. Jean-Claude Henrard returned to Paris in October 1972 following a year with the Section as guest worker under the sponsorship of the Fulbright program.

The work of the Section has continued to focus primarily on the factors associated with the development of diabetes mellitus and, in particular, the associated vascular complications.

The extent of diabetes among Southwestern Indian populations has been further investigated by a study of adult Papago Indians. The results of glucose tolerance testing indicated that about 35% of the males and 50% of the females aged 35 years and over had glucose intolerance diagnostic of diabetes mellitus. These rates are slightly lower than those found in the Pima Indians but emphasize the extraordinarily high prevalences of diabetes found among the desert dwelling American Indian populations. The rates in the Papago who are distantly genetically related to the Pima Indians tend to support the concept that the high prevalence in the Pima Tribe is not simply the result of genetic isolation. The distribution of postload plasma glucose levels in the Papago was found to be bimodal, confirming the type of distribution first described among the Pima Indians. Studies of the San Carlos-Apache Indians will shortly be made in an attempt to offer a critical test of the genetic versus environmental hypothesis as a cause for the aggregation of diabetes among the Southwestern Indian people.

Among the Pima Indians diabetic nephropathy has been shown to be strongly correlated with the presence of hypertension. This finding together with the previously described associations of diabetic retinopathy and hypertension indicates that hypertension is probably related to the development of nephropathy rather than being a sequela of nephropathy. These data indicate the need for a controlled clinical trial of antihypertensive measures among diabetics to determine if the onset and rate of development of the specific vascular complications of diabetes may be reduced or prevented by such measures.

Analysis of electrocardiographic findings and autopsy results confirm the low prevalence and incidence of myocardial infarction among Pima Indians in spite of the high prevalence of diabetes mellitus and obesity. As the Pima Indians with diabetes appear equally susceptible to the complications of nephropathy and retinopathy as other diabetes clinic populations, these findings suggest that the pathogenesis of major arterial disease may differ from that of microvascular disease. This hypothesis will be pursued by determining the associations of other major vessel diseases and diabetes among the Pima Indian population.

Evidence of medial arterial calcification has been found to be related to the presence of glucose intolerance but the rates and associations vary quantitatively in males and females indicating that other factors play a part in determining the appearance of this complication of diabetes.

Studies of Pima Indians who have developed diabetes mellitus during the course of the NIAMDD study have indicated that persons with hypertension and excessive obesity are more prone to show marked deterioration of glucose intolerance than others. Further factors which characterize the prediabetic subject will be sought.

The Section has recently conducted a study of glucose tolerance among the inhabitants of St. Paul and St. George Islands in Alaska. Five years ago the inhabitants of these Islands suffered a well documented outbreak of Coxsackie B_4 infection. Those who were affected were identified by the collection of both pre-epidemic and post-epidemic sera by the Center for Disease Control. Since Coxsackie B_4 has been specifically implicated as a possible cause or precipitating factor in diabetes mellitus, glucose tolerance tests were conducted on members of the population who had been previously infected with Coxsackie B_4 five years ago and on control subjects who specifically escaped infection at that time. Although the study has just been completed and results are not yet available this project should provide a critical test of the hypothesis that Coxsackie B_4 infection may be a cause of diabetes mellitus.

An investigation of the frequency of hyperostotic spondylosis and its associated factors was conducted among the Pima Indian population. This little recognized form of spinal arthritis was found to be extremely common among the Pima Indians and in view of the purported associations of this disease with diabetes it was anticipated that strong relationships with the presence of diabetes would be found. While some association was found the strength of the association was much lower than that which had been suspected on the basis of reports from other centers. Hyperostotic spondylosis was found to not only involve the thoracic spine but was found equally frequently in the lumbar spine and about half as frequently in the cervical region. The evidence of cervical spine involvement confirms a recent case report. The disease was found to affect males much more frequently than females and its primary manifestations were found to be a limitation of movement in the spine. Because the disease was not associated with pain it was found to be an unusual cause for patient symptoms. The clinical significance of the disorder lies primarily in recognizing these manifestations as distinct from those of ankylosing spondylitis and disc degeneration of the spine.

Members of the Section have participated in the design of the multicenter study to determine the efficiency and safety of chenodeoxycholic acid in the dissolution of human gallstones. It is anticipated that the proposed trial will start in the forthcoming year and that many of the Pima Indians previously identified with asymptomatic cholelithiasis will be recruited for participation in this controlled clinical trial.

The analysis of the epidemiologic data collected over the past five years has continued to be hampered by the availability of only a single computer programmer. This problem has restricted the output of the Section considerably since the section is almost totally dependent on the availability of computer programming personnel to retrieve data in useable form.

METABOLIC DISEASES EPIDEMIOLOGY UNIT

The metabolic diseases epidemiology unit has continued to pursue the identification and isolation of anti-thyroid compounds which develop during the culture of a variety of micro-organisms. Recent work has focused on the production of anti-thyroid compounds derived from cultures of Esherichia coli. These compounds have now been produced in large quantities and successfully concentrated. It is expected that the availability of a more concentrated and pure form of this material will enable its further characterization and identification.

Serial No. NIAMDD-EFS-1(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Prevalence of Diabetes in Indian Populations in the Southwestern United States

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Stephen E. Dippe
Dr. Joseph A. Ingelfinger

Cooperating Units: Indian Health Service (PHS)

Man Years:

Total: 0.5
Professional: 0.2
Others: 0.3

Project Description:

Objectives:

1. To determine the prevalence of diabetes among various native populations in the Southwestern United States.

2. To formulate and test hypotheses which may explain the high frequency of diabetes mellitus among some of these populations.

Methods:

Random samples of total populations will be examined by means of standardized tests of glucose tolerance to enable the prevalence of glucose intolerance to be determined. Other examinations will be performed to document the frequency of other disorders when appropriate.

Major Findings:

A survey was conducted to determine the frequency of glucose intolerance among the Papago Indians residing in the Sells and Gu-Achi districts of the Papago reservation in southern Arizona.

A two-hour post 75 gram oral carbohydrate load plasma glucose level, urinalysis, height, weight, and short clinical history were obtained from 370 Papago Indians aged 35 and over.

Subjects were considered to be diabetic if their two-hour plasma glucose was 200mg/100ml or greater, or if they were taking insulin or oral hypoglycemic agents. Overall, 29% of the males and 39% of the females were found to be diabetic. In the Gu-Achi District, where the 200 subjects represented an estimated 75% completion rate, 34% of the males and 40% of the females were diabetic. These rates are not significantly different from the prevalence found in the genetically and linguistically related Pima Indians. The frequency distribution of the two-hour post load plasma glucose levels in the Papago Indians was found to be bimodal and similar to the distribution in the Pima Indians.

PREVALENCE OF HYPERGLYCEMIA* AMONG SOUTHWESTERN INDIANS

	Males				Females			
Age (years)	<35		≥35		<35		≥35	
Tribe	No. Exam.	Percent Positive	No. Exam.	Percent Positive	No. Exam.	Percent Positive	No. Exam.	Percent Positive
White River Apache	--	--	109	3.7	--	--	159	18.2
Navajo	21	0.0	28	7.1	36	2.8	27	18.5
Washoe	16	0.0	34	8.8	30	3.3	32	25.0
Paiute	39	0.0	27	18.5	38	2.6	27	33.3
Upland Yumans	52	0.0	144	21.2	93	1.8	169	38.6
Zuni	87	2.9	140	17.2	312	3.8	152	45.4
Cocopah	15	6.7	35	25.7	29	6.9	44	40.9
Papago	--	--	170	36.5	--	--	195	48.2
Pima	921	4.3	437	41.0	1098	6.5	461	58.1

*Plasma glucose level ≥160/100ml two hours post 75G carbohydrate load or ≥180mg/100ml one hour post load.

Significance of Research:

The prevalence rates of diabetes mellitus among the various Indian tribes in the Southwestern United States appear to be substantially unrelated to their genetic origins. This finding suggests that the high prevalence of diabetes observed in many may represent an adaptation, with a possible genetic basis, to certain types of environment, rather than simply an expression of a common genetic background.

Proposed Course:

Glucose tolerance testing will be continued among other Indian tribes residing in the Southwestern United States in order to define further the possible reasons for the great variation in the prevalence of diabetes mellitus in this area.

Honors and Awards: None

Publications:

Bartha, G.W., Burch, T.A. and Bennett, P.H.: Hyperglycemia in Washoe and Northern Paiute Indians, Diabetes, January, 1973, Vol. 22, No. 1, pp. 58-62.

Serial No. NIAMDD-EFS-2(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Prospective Study of the Natural History of Diabetes Mellitus in the Gila River Indian Community

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Stephen E. Dippe
Dr. Stephen A. Kamenetzky
Dr. Joseph A. Ingelfinger
Dr. Peter J. Savage
Miss Phyllis E. Sperling
Dr. Max Miller
Dr. Arthur G. Steinberg
Dr. Norman B. Rushforth
Dr. Elmer J. Ballintine
Dr. Irving M. Liebow
Dr. Frederick A. Rose

Cooperating Units:
Indian Health Service (PHS)
Case Western Reserve University
National Eye Institute

Man Years:

Total:	9.3
Professional:	3.4
Others:	5.9

Project Description:

Objectives:

1. Determine the prevalence and incidence of chemical and clinical diabetes mellitus among those five years of age and over on the Gila River Indian Reservation.

2. Evaluate factors associated with the progression from prediabetic state to chemical diabetes mellitus and to clinical diabetes.

3. Study the association between diabetes and rate of appearance and progression of vascular complications.

4. Investigate influence of tribe, age, obesity, parity, and other factors on changes in glucose tolerance and their relationship to diabetes mellitus.

Methods:

1. The following tests and examinations will be repeated at two yearly intervals on all Indians five years of age or more living on the Sacaton Service Unit portion of the Gila River Reservation:

a. Clinical interview
b. Physical examination
c. Modified glucose tolerance test
d. Fundus photographs
e. Electrocardiogram
f. Tests for vibratory sensation threshold
g. Soft tissue radiographs for vascular calcification

2. Some of the above tests and examinations will also be done on Indians of other tribes or living under different environmental conditions for comparison.

Major Findings:

Renal Disease and Hypertension: It is known that Pima diabetics have a high prevalence of renal disease in contrast to their non-diabetic tribesmen. The correlation of elevated blood pressure with the prevalence of renal disease was examined by measuring proteinuria, and systolic (SBP) and diastolic (DBP) blood pressure in 318 diabetic and 540 non-diabetics aged 15-64.

In the diabetics, the frequency of proteinuria ($\geq$30mg/100ml) increased with rising SBP ($p<0.001$) and DBP ($p<0.05$). In all duration groups (<5,5-9,$\geq$10 years), subjects with SBP <140mmHg had significantly less proteinuria ($p<0.025$) than subjects with higher blood pressure values. Similarly the prevalence of proteinuria in those with DBP <90mmHg was also lower in each duration category, but this difference was statistically significant only in those with duration of 10 or more years ($p<0.01$).

In the non-diabetics, there was no correlation between increasing SBP and the prevalence of proteinuria. DBP was correlated with proteinuria only in subjects aged 25-44 when examined ($p<0.05$).

Pima diabetics had a prevalence of proteinuria 3.3 times that found in non-diabetics ($p<0.001$). However, the prevalence of proteinuria in diabetics with SBP <140mmHg was not significantly different from that found in non-diabetics, suggesting that the excess proteinuria in the diabetics occurs primarily in subjects who also have elevated SBP.

Since hypertension is not infrequent in diabetics and even moderate elevation of systemic blood pressure is strongly correlated with an increased prevalence of proteinuria, it is possible that careful control of blood pressure may delay the onset or slow the progression of nephropathy in subjects with diabetes.

RELATIONSHIP OF BLOOD PRESSURE TO PROTEINURIA IN PIMA DIABETICS AGED 25-64

	DURATION OF DIABETES (YRS)					
	0-4		5-9		≥10	
Blood Pressure	No. Exam.	Percent Positive	No. Exam.	Percent Positive	No. Exam.	Percent Positive
Systolic:						
<140mmHg	101	6.9	27	3.7	14	14.3
≥140mmHg	100	21.0	40	40.0	30	55.6
Diastolic:						
<90mmHg	138	10.9	48	22.9	27	25.9
≥90mmHg	63	20.6	19	31.6	23	65.2

Coronary Heart Disease: Major Q wave changes (Minnesota Code I-1, I-2) were found significantly more frequently among the male Pimas than among the females, and also more frequently among Pimas over 60 years of age. Diabetic Pimas had almost twice the rate of Q waves as non-diabetic Pimas, but this difference was not significant.

Analysis of the twelve lead electrocardiograms from 701 Pima Indians, aged 40 years and over, has suggested that major T wave inversion (V-1, V-2 as coded by the Minnesota Code) may not be a good measure of coronary heart disease in Pima Indians. Other reports have suggested that the significance of T wave inversion may vary from race to race and even between the sexes.

The age and sex standardized prevalence of major Q wave changes among the Pimas was approximately one-half that reported from the mostly white and non-diabetic population of Tecumseh, Michigan, but this difference also was not significant.

PREVALENCE OF Q WAVES AMONG THE PIMA INDIANS: RATE/100
(Where age groups are combined the rates are standardized to the entire Pima population)

	MALES			FEMALES		
AGE GROUP	40-59 YEARS	60+ YEARS	Both	40-59 YEARS	60+ YEARS	Both
Major Q Waves (MC I-1,I-2)						
Non-Diabetic	0.8	4.2	2.1	0.0	0.0	0.0
Diabetic	1.3	5.4	2.9	0.0	2.7	1.9
Both	1.0	4.7	2.4	0.0	1.7	0.6

Characteristics of Pima Indians Prior to the Development of Marked Glucose Intolerance: Nine-hundred and fifty Pima Indians aged 15 to 64 who had an initial 2 hour post 75 gm oral carbohydrate load plasma glucose level of less than 200mg/100ml were retested one to four times during 1966-72. A subject's expected retest two-hour glucose value was computed from his initial value and age and sex specific regression equations relating retest to initial glucose values.

The distribution of the differences between actual and expected retest glucose values was bimodal. Most subjects had all retest glucoses falling in the first component of this bimodal curve. Sixty-three individuals had at least one retest glucose in the second component, and were considered to have undergone a significant deterioration in glucose tolerance. All had at least one two-hour glucose >275mg/100ml, and two-thirds had values >400mg/100ml.

Two to 4 years prior to the onset of marked glucose intolerance, these 63 subjects were on the average 25% more obese than Pimas whose two-hour glucose values remained <200mg/100ml ($p<0.01$). They also had higher mean systolic and diastolic blood pressures ($p<0.05$). There was a modest, but statistically not significant, decrease in glucose tolerance prior to the development of marked glucose intolerance. There were sixteen subjects with two exams prior to the development of marked glucose intolerance. Fourteen of the sixteen had two-hour plasma glucose values which were 2 to 80mg/100ml lower on their first exam than their second exam. This suggests that in the Pima, the appearance of marked glucose intolerance may be preceded by a period of slowly decreasing glucose tolerance.

Arterial Calcification: Among Pima Indians arterial calcification is very common and related to sex, age, and two-hour plasma glucose. With rare exception medial arterial calcification was predominant as seen on x-rays of feet, hands, calf, thigh, and pelvis.

Results were attained on 1138 persons ages 25 years and over of whom 531 were male and 607 were female. Following a 75 gm carbohydrate load, 31% of the females and 40% of the males had two hour plasma glucoses above 200mg/100ml.

Seventy percent of males above 54 years had developed arterial calcification regardless of two-hour plasma glucose. Among males the age of onset of arterial calcification occurred 10-20 years earlier when the two-hour plasma glucose was 200mg/100ml or greater. Also, those males with plasma glucoses between 140 and 200mg/100ml developed calcification approximately 5-10 years earlier than those with lower glucose levels.

Females had one-half the overall frequency of vascular calcification as males. However, for each age group a higher frequency of arterial calcification occurred in those with a two-hour plasma glucose greater than 200mg/100ml. The females showed increasing frequency of calcification with increasing two-hour plasma glucose so that above 400mg/100ml male and female frequency of arterial calcification were the same. Females whose two-hour plasma glucose ranged between 140-200mg/100ml did not show any increased arterial calcification. Males are more susceptible to the development of arterial calcification and are more sensitive to slight elevation of two-hour plasma glucoses. A major finding is that regardless of the plasma glucose, 70 percent of males beyond 54 years of age developed arterial calcification suggesting that some persons are protected from arterial calcification even with high glucose levels. Females with normal glucoses on the other hand had almost no arterial calcification but showed a much higher prevalence when glucose levels were elevated. However, higher two-hour plasma glucose levels were required to match the same frequency of arterial calcification as found in males.

PERCENT WITH VASCULAR CALCIFICATION

	2-Hour Plasma Glucose (mg/100ml)					
	<140		140-199		>199	
AGE	M	F	M	F	M	F
25-34	4	0	0	0	5	9
35-44	8	3	15	3	32	19
45-54	19	3	33	5	73	20
55-64	68	14	85	15	76	52
65-74	80	43	63	30	78	77

Serum Immunoreactive Insulin (IRI in Pima Indians: Analysis was completed in over 300 Pima Indians using the two-hour glucose and insulin values. Two-hour plasma glucoses were grouped in 15-25 mg intervals and the mean delta IRI was determined for each glucose group. Delta insulin represents the difference between the two-hour IRI and the fasting IRI.

The mean delta IRI remained constant up to a two-hour plasma glucose of 114mg/100ml. The mean delta IRI then rose linearly and proportionately to the two-hour plasma glucose up to approximately 140mg/100ml. The mean delta IRI between the two-hour glucose range of 140 to 180mg/100ml remained somewhat level. However, the delta IRI did not continue to rise in spite of increasing glucoses. Thus, the insulin/glucose ratio showed a continued reduction in this glucose range. Above a two-hour plasma glucose of 180mg/100ml, the delta IRI decreased linearly and proportionately to the glucose. After a two-hour plasma glucose of 430mg/100ml, the delta IRI changed very little.

This data suggests that with a slight elevation of the two-hour plasma glucose, the insulin responds appropriately to the elevated glucoses and that it may represent a delay in insulin release earlier in the glucose tolerance test. The data also infers that marked insulinopenia occurs only with severe carbohydrate intolerance. Finally a period of hyperinsulinism may preceed the development of severe diabetes.

Obese subjects followed the same pattern as non-obese except that the mean delta IRI was higher for each 2 hour plasma glucose group. Overall analysis of weight did not show any significant difference in the various two-hour plasma glucose group.

Relationship of two-hour Plasma Glucose to Δ Immunoreactive Insulin (2-hr. IRI-Fasting IRI)

No.	2-hr. Plasma Glucose mg/100ml	ΔIRI μU/ml ± SEM
12	<100	65.6±13
16	100-114	63.4±13
18	115-129	117.2±10
21	130-144	164.7±41
24	145-159	142.2±17
18	166-174	144.8±20
19	175-189	155.2±24
18	190-204	122.2±22
11	205-224	112.3±18
18	225-244	81.4±14
14	245-264	89.4±19
17	265-284	82.9± 8
10	285-304	32.9±10
12	305-329	57.3± 8
15	330-354	44.2±11
22	355-379	20.5± 3
10	380-404	29.9±10
8	405-429	17.4± 5
9	430-454	4.4± 3
17	455-479	6.9± 3
19	>480	3.3± 2

Significance of Research: The vascular complications of diabetes mellitus are responsible for the majority of the morbidity and mortality associated with diabetes mellitus. The relationship of hypertension to nephropathy and as previously shown retinopathy, appears to represent an important factor in the pathogenesis of the retinal changes, and one whose effect is potentially amenable to alteration by safe medical means.

The observation of the close association of renal disease and retinal abnormalities suggests that factors influencing the progression of one of these complications might well influence the other. However, the presently applied methods of investigation for renal disease are relatively insensitive and non-specific, and it seems probable that the application of more quantitative, sensitive, and discriminating methods for the evaluation of renal function could lead to a better understanding of factors related to the development and progression of diabetic nephropathy.

The cross-sectional analysis of the relationship of insulin to glucose suggested that in development and worsening of diabetes mellitus, a period of hyperinsulinism preceeds insulinopenia. This phenomenon appears to be independent of weight and age. Thus peripheral interference of insulin or perhaps impedance of insulin release to a glucose challenge occurs early in the course of the disease.

Medial arterial calcification is found to be very common in the Pima Indians even though other large vessel disease is minimal. The arterial calcification is found more frequently in males and is closely associated with the degree of carbohydrate intolerance.

Proposed Course:

The prospective study of diabetes and its complications will be continued. The frequency and characteristics of renal functional abnormalities will be determined using sensitive methods for the measurement and characterization of proteinuria and applied to subjects with subclinical evidence of renal disease by conventional definitions, i.e., subjects without proteinuria. The early recognition and natural history of proteinuria in the diabetic may lead to the recognition of important modifying factors.

The blood lipids of the Pima Indian population will be characterized to determine if their low prevalence of coronary heart disease is related to unusual lipid characteristics.

Multiple factor analysis on a number of diabetic complications will be carried out to identify long duration diabetics without any complications. Comparative data and further studies will be carried out to identify some features of the group which appear protective.

Characterization of prediabetes will be carried out using past and present data as well as evaluating insulin data.

Since a large number of prediabetic subjects have now been identified additional analyses will be performed to try to identify any factors which lead to the development of diabetes mellitus in this high risk group.

Honors and Awards: Dr. Arthur Dorf, J.D. Lane Award of USPHS.

Publications:

Bennett, P.H. Epidemiologic Studies of Diabetes Mellitus: Pan American Health Organization, Epidemiologic studies and clinical trials in chronic diseases, Washington, D.C., Pan American Health Organization, 1972 (PAHO Scientific Publication No. 257.)

Serial No. NIAMDD-EFS-3(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Prospective Study of the Natural History of Arthritis and Rheumatism in the Gila River Indian Community

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Thomas A. Burch
Dr. Stephen E. Dippe
Dr. Peter J. Savage
Dr. Stephen A. Kamenetzky
Dr. J. A. Ingelfinger
Miss Phyllis E. Sperling

Cooperating Units:
Indian Health Service (PHS)
Hawaii State Health Department

Man Years:

Total:	1.4
Professional:	0.4
Others:	1.0

Project Description:

Objectives:

1. Determine the progression of clinical, radiological and serological features of joint diseases affecting the Gila River Indians with particular attention to rheumatoid arthritis, osteoarthrosis, ankylosing spondylitis, and gout.

2. Determine environmental and genetic factors associated with development and progression of arthritis and rheumatism.

3. To compare and contrast the clinical course of rheumatoid arthritis in sero-positive individuals with that of sero-negative arthritics.

4. Determine the predicative and prognostic significance of rheumatoid factor in relation to the clinical course of rheumatoid arthritis.

Methods:

1. The following tests and examinations will be repeated on all Indians five years of age or over, living on the Sacaton Service Unit portion of the Gila River Reservation.

   a. Clinical interview.
   b. Physical examination.
   c. Radiographs of hands, feet, and cervical spine. Males over age 14 and females over age 44 will have a pelvis film taken.
   d. Laboratory determinations: rheumatoid factor, uric acid.

2. The data from the above examinations and tests as well as demographic, pedigree, environmental information, and data on other conditions and diseases will also be collected.

Major Findings:

Hyperostotic Spondylosis: Hyperostotic Spondylosis (HS) is a spinal bone disease characterized by the development of bony bridges between the vertebral bodies and by the overlaying of newly formed bone on the anterior and lateral aspects of the vertebral bodies.

Radiographs of the spine of an age-sex stratified random sample of 600 Pima Indians were evaluated for the characteristic features of HS and classified according to whether each characteristic was present in moderate or severe degree, absent or possibly present. Depending on the number and severity of these characteristics in each region of the spine the presence or absence of definite HS was determined.

HS occurred equally frequently in the thoracic and lumbar spine and less than half as frequently in the cervical region. Males were found to show definite evidence of the disease in the thoracic spine three times as frequently as females and ten times as often in the lumbar spine. Strict prevalence estimates were difficult to ascertain as there were many subjects with evidence of probable HS in whom differentitation from other forms of spinal disease, especially degenerative disc disease, proved difficult. However, 25 percent of the adult males had definite HS.

A sample of those with definite radiologic evidence HS were compared with age-sex matched controls to determine associations of HS with other characteristics. The findings are summarized in the table.

Other Joint Findings in Subjects with and without Hyperostotic Spondylosis

	With HS % with Changes	Controls % with Changes	(P) Significance
Limitation of cervical spine	39	14	<0.02
Limitation of dorsolumbar spine	49	11	<0.001
Pain on motion of spine	11	6	NS
Heberden's nodes	34	7	<0.02
Radiologic evidence of osteoarthrosis			
in hands	90	52	<0.001
in feet	54	24	<0.02
Radiologic evidence of disc degeneration			
in cervical spine	44	50	NS
in dorsal spine	23	5	NS
in lumbar spine	48	58	NS

It was concluded that spinal hyperostosis is a disease predominantly affecting males and is associated with peripheral evidence of joint disease with degenerative or hypertrophic characteristics, and limitation of the spine as its major clinical manifestation.

Previous reports have suggested that hyperostotic spondylosis is strongly associated with diabetes mellitus. Because of the high prevalence of both disorders among the Pima Indians the interrelationships were examined.

Glucose Intolerance in Males with HS and Controls
(Two-hour post load plasma glucose >160mg/100ml

Age	Cases	Controls
45-54	67%	33%
55-64	36%	18%
65-74	50%	33%
TOTAL	50%	28% p<0.05

As shown in the table a moderate, but significant, association was found between the two diseases, but the magnitude of the association indicated that it was far from specific. This suggests that the metabolic defect leading to hyperostotic spondylosis is not found exclusively in the diabetic subject.

Significance:

Most of the previous descriptions of hyperostotic spondylosis have been hampered in their ability to define meaningful associations because of the methods of case selection.

The present study has shown that the hyperostotic spinal disease is associated with other peripheral joint disease and that while it occurs more frequently in the male diabetic it occurs quite frequently in non-diabetic subjects also.

Proposed Course:

No further studies of hyperostotic spondylosis are planned in the immediate future, but the long term goals of the overall study of the natural history of arthritis will be continued through the continued biennial examinations.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-EFS-4(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Southwestern Indian Gallbladder Study

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:	Dr. J. A. Ingelfinger Dr. S. M. Grundy Dr. Donald M. Small
Cooperating Units:	Indian Health Service (PHS) Boston University

Man Years:

Total:	1.0
Professional:	0.5
Other:	0.5

Project Description:

Objectives:

To determine whether cholesterol gallstones may be dissolved effectively and safely by the oral administration of bile salts.

Methods:

1. Adult Indian subjects who have asymptomatic gallstones will be identified.

2. Such subjects will be asked to participate in a double blind controlled study of the effect of oral administration of bile salts in producing the dissolution of the gallstones over a two-year period.

Major Findings:

None. All activity this year has been directed towards the further development of a detailed study protocol for the multicenter cooperative trial. It is expected that the study will commence in the coming year.

Significance of Research:

Chenodeoxycholic acid has been demonstrated to be an effective means of dissolving radiolucent gallstones in persons with a functioning gallbladder. The safety and side effects of this mode of therapy have not yet been adequately documented in a large patient series, nor in American Indians. The multicenter cooperative trial will result in such information and result in the development of the specific indications for bile salt therapy versus cholecystectomy in the treatment of cholelithiasis.

Proposed Course:

To conduct the trial in accordance with the collaborative study protocol.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-EFS-5(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Prospective Study of the Complications and Outcome of Diabetic and Prediabetic Pregnancies in the Gila River Indian Community

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Stephen E. Dippe
Dr. Peter J. Savage
Dr. S. A. Kamenetzky
Dr. Max Miller

Cooperating Units:
Indian Health Service (PHS)
Case Western Reserve University

Man Years:

Total:	0.7
Professional:	0.2
Others:	0.5

Project Description:

Objectives:

To determine whether pregnancies in prediabetic women are associated with excessive complications during pregnancy or with excessive mortality or morbidity in the offspring.

Methods:

The course and outcome of pregnancies in all female residents of the Gila River Indian Community (Sacaton Service Unit area) are followed and recorded in a standardized manner.

All patients in the prenatal clinic of the Sacaton Hospital receive a modified glucose tolerance test during the third trimester of their pregnancy. These patients receive another modified glucose tolerance test 24-48 hours post partum and are retested at approximately two yearly intervals to document the development or lack of development of diabetes. Infants will be examined to determine the presence of congenital anomalies and other characteristics, and data related to mortality and morbidity will be compiled. At age five years the children will be re-examined to detect any further abnormalities which may have been inapparent at birth.

Major Findings:

None. Further analysis has been deferred pending collection of additional data.

Significance of Research:

In view of the high prevalence of diabetes in the Pima Indians, studies in the Gila River Community provide a unique opportunity to evaluate the outcome of both prediabetic pregnancies and pregnancies with mild carbohydrate intolerance. Retrospective studies of the course of prediabetic pregnancies have to date produced conflicting results. Although it is well established that patients with severe glucose intolerance, especially those with diabetes of long duration and significant microvascular complications, have an increased prevalence of fetal mortality and maternal morbidity, it is not certain whether treatment will improve the outcome of those pregnancies in which there is mild glucose intolerance. Limited prospective studies of the benefit of treating pregnant women with mild glucose intolerance have so far yielded conflicting results. With the recent adoption by the Indian Health Service of more stringent criteria for the treatment of mild carbohydrate intolerance in pregnancy it is hoped that this study will be able to add to the evaluation of this problem by monitoring the impact of these therapeutic changes on the outcome of pregnancies on the reservation.

Proposed Course:

The pregnancy study will continue in conjunction with the study of the natural history of diabetes mellitus in the Gila River Indian Community. The number of pregnancies recognized to be prediabetic will increase as the mothers studied develop diabetes mellitus.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-EFS-6(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Gila River Indian Community Autopsy Study

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Stephen E. Dippe
Dr. Joseph Likos
Dr. Stephen A. Kamenetzky
Dr. Joseph A. Ingelfinger
Dr. Peter J. Savage
Dr. Philip M. LeCompte
Dr. Max Miller

Cooperating Units:
Indian Health Service (PHS)
Veterans Administration
Faulkner Hospital, Boston Massachusetts
Case Western Reserve University

Man Years:

Total:	0.8
Professional:	0.2
Others:	0.6

Project Description:

Objectives:

1. To determine the causes of death, and associated disease processes in members of the Gila River Indian Community being followed in prospective studies on arthritis, diabetes mellitus, cholelithiasis, etc.

2. To ascertain and evaluate the histopathological changes in tissues and cells as they relate to diabetes mellitus. To assess their importance and relationship to clinical findings in members of the Gila River Indian Community upon whom autopsies are performed.

3. Evaluate factors and conditions associated with increased or decreased mortality rate in the Gila River Indians.

Major Findings:

Myocardial Pathology: The results of the 101 autopsies of Pima Indians obtained in the last 7 years were reviewed. Eleven cases of myocardial infarction were found. The age and sex distribution of infarcts at post mortem examination confirms findings from prevalence data that coronary heart disease is a disease of older Pima Males. No significant association of diabetes with myocardial infarct could be demonstrated. Yet comparison of the frequency of infarct at autopsy with the rates reported from other race and location groups suggests that the Pimas have a low frequency of infarct despite their high prevalence of diabetes mellitus.

AGE ADJUSTED FREQUENCY/100 of Myocardial Infarction at Autopsy from Various Countries as Reported by Strong, et al, (1) and in the Pima Indians

Location (Race)	MALES Aged 35-69	FEMALES Aged 45-69
New Orleans (White)	31	21
Oslo (White)	32	20
New Orleans (Negro)	15	11
Puerto Rico (White)	9	17
Guatemala City (American Indian, White)	8	--
Santiago (White)	4	--
Pima Indian	7	0.0

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(1) Strong, J.P., Solberg, L.A., Restrepo, C.: Atherosclerosis in persons with coronary heart disease. Lab.Invest. 18: 527-537, 1968.

Renal Disease: Histologic data were obtained from the analysis of postmortem kidney material from 105 Pima Indians aged 15 and over. Clinical records, reviewed subsequently, showed that 40% were diabetic, a prevalence similar to that for the Pima population as a whole.

Diffuse glomerulosclerosis was present in 65% of the diabetics and in only 3% of the non-diabetics. Nodular glomerulosclerosis, a lesion felt to be virtually specific for diabetic renal disease, was present in 53% of subjects with diabetes and only in those with a history of diabetes. Exudative glomerular lesions was also found in over 40% of the diabetics.

One-third of the diabetics had no evidence of either glomerulosclerosis or exudative changes. The mean duration of diabetes in this group was 5.5 years in contrast to 12.1 years for those with evidence of nephropathy.

Moderate to severe arteriolar changes were found in 77 percent of the diabetics and only 15% of the non-diabetics. Histologic changes characteristic of pyelonephritis were seen in 16% of subjects with diabetes and 5% of the normals. Other kinds of renal disease were rare, with only a single case of glomerulonephritis in the entire series.

The results of the pathology study are summarized below:

		Diabetic	Non-diabetic	
Number of patients examined	105	43	62	
Males	61	25	36	
Females	44	18	26	
Mean Age (years)		62.3	59.3	
Percent with:				Significance (p)
moderate-severe diffuse glomerulosclerosis		65.1	3.3	<0.001
nodular glomerulosclerosis		55.8	0.0	<0.001
exudative glomerular lesions		44.1	0.0	<0.001
arteriolar changes		76.7	14.5	<0.001
pyelonephritis		16.3	5.0	N.S.
glomerulonephritis		0.0	1.7	N.S.

Significance of Research:

This study will result in a more precise clinical-pathologic correlation than has been previously possible in diabetes mellitus. It will enable the prognostic significance of a variety of clinical findings to be assessed. The obvious advantages of an epidemiologic approach will be realized and a more complete understanding of the significance of glucose intolerance and its sequelae will result.

The relatively low frequency of infarct at death supports the prevalence data in suggesting that the Pima have a relatively low rate of myocardial infarction despite their high prevalence of diabetes mellitus. Further study of the incidence of coronary disease in the Pima may support these findings and determine what factors in diabetic and non-diabetic Pimas predispose to coronary disease.

Because classical diabetic nephropathy is common in Pima subjects, this population is ideal for the study of the natural history of diabetic renal disease and the effects of various risk factors on its progression and development.

The observation of a high prevalence of histologic evidence of the specific renal changes of diabetes will enable this disorder to be characterized more fully both clinically and histologically.

Proposed Course:

The systematic collection of tissues will continue. As sufficient specimens become available for special study, detailed examinations of various tissues will be made. Further studies of the kidney, using electron microscopy are planned.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-EFS-7(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Coxsackie B_4 Virus Infection and its Relationship to Diabetes Mellitus

Previous Serial Number: None

Principal Investigator: Dr. Stephen E. Dippe

Other Investigators:	Dr. Peter H. Bennett Dr. Donald Dippe Dr. James Maynard Dr. Ken Burquist Dr. Max Miller
Cooperating Units:	Indian Health Service Center for Disease Control Case Western Reserve University

Man Years:

Total:	0.4
Professional:	0.2
Others:	0.2

Project Description:

Objectives:

1. An investigation of the prevalence of diabetes mellitus was undertaken in the Pribilof Islands inhabitants because of the reported occurrence of a high frequency of recent Coxsackie B_4 infection among newly diagnosed diabetics elsewhere. Five years ago a well documented outbreak of Coxsackie B_4 infection occurred on the Pribilof Islands attacking approximately one-half of the population, which had not been previously exposed to this infection.

2. To determine the prevalence and incidence of diabetes mellitus five years after an epidemic caused by Coxsackie B_4 virus.

3. To evaluate the relationships of serum lipids and blood pressures to diabetes and cardiovascular abnormalities.

Methods:

1. The following tests and examinations were completed on all residents of the islands age 10 and above:

a. Clinical interview
b. Physical examination
c. Electrocardiogram
d. 75 gm carbohydrate load tolerance test
e. Laboratory analysis for various virus studies and lipid studies.

2. All deaths occurring in the five year period are to be analyzed as to predisposing factors and cause of death.

Major Findings:

None. Data collection has just been completed.

Significance of Research:

Coxsackie B_4 virus has been implicated as a possible etiologic agent in the development of diabetes mellitus. These studies should lend considerable information concerning the importance of this virus in the development of diabetes mellitus in a population that had no experience with this virus prior to the epidemic five years ago.

Proposed Course:

If a relationship between previous Coxsackie B_4 infection and diabetes is found the population will be examined for carbohydrate tolerance, virologic titers, lipid, and insulin levels, and EKG findings at suitable intervals. A continuous ongoing study of all deaths that occur on the islands will be performed.

Honors and Awards: None.

Publications: None.

Serial No. NIAMDD-EFS-8(c)
Epidemiology & Field Studies Branch
Metabolic Diseases Epidemiology Unit
Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Experimental and Field Studies of Iodine Metabolism

Previous Serial Number: NIAMD-EFS-7(c)

Principal Investigator: Robert L. Vought, M.D., M.P.H.

Other Investigators: Freddie A. Brown, B.S.

Cooperating Units: Laboratory of Nutrition and Endocrinology, Clinical Endocrinology Branch, NIAMDD

Man Years:

Total:	3
Professional:	2
Other:	1

Project Description:

Objectives: The long-term objectives of the program are: (a) to examine closely the principal environmental factors associated with prevalence of goiter and (b) to study the nature of the metabolic defects responsible for the disease.

Methods Employed: Accomplishment of the objectives has involved bench laboratory research, clinical research and collaborative field studies.

During the current year a satisfactory assay for antithyroid compounds has been developed. It is an *in vivo* assay in the rat and it has been tested against known goitrogenic compounds; its sensitivity and reliability have been determined, also.

Major Findings: During the current year it has been shown that *Penicillium* sp. and *Escherichia coli* consistently produce antithyroid and possibly goitrogenic compounds. The latter was selected for more intensive study because of the ease and rapidity with which it is grown. Several strains of *E. coli* have been studied and all have shown ability to produce antithyroid compounds. Cell-free filtrates of broth cultures have also been fractionated by ultra-filtration and by precipitation with ammonium sulfate and the fraction which shows the greatest activity has been discovered. It has been determined that this partially purified fraction contains as much activity as 100 times the activity found in crude material.

OFFICE OF PUBLIC INFORMATION ACTIVITIES

Summary

Fiscal Year 1973

The Information Office conducts the Institute's public information and educational programs, and prepares the bulk of the regular and special program reports required of the Institute by the Congress, and by NIH and HEW.

Heavy demands are placed on the small information staff to report on the Institute's varied and complex activities, which have increased both in scope and depth in recent years. There are not one but several publics which must be reached with NIAMDD information. They can be grouped as follows: The general public, the professional public, the Governmental public and the Congressional public. The media used fall into three broad categories, the mass media, specialized media and, individual communications.

The following is a review of the performance of the Office of Public Information during the past fiscal year.

Press Activities:

It is the policy of the National Institutes of Health to prepare and issue news releases on newsworthy subjects about its research activities and programs. In this regard, the NIAMDD Information Office staff this past fiscal year prepared information on a wide variety of topics. Characteristic press releases included, for example, the awarding of contracts to medical research centers for studies of Crohn's disease, the pinpointing of the biochemical defect of Hurler's syndrome, the announcing of a new "alerting" publication, Index of Dermatology, that will list the latest dermatology-related scientific papers and publications in all languages from around the world; and the publicizing of Dr. Roger C. Guillemin's delivery of the 1973 National Institutes of Health Lecture.

Television and Radio:

The information arm of the Office of the Director has participated with the central NIH Information Office in the production of projects designed to bring the Institute to the attention of the public. These projects include various interview and discussion shows. A number of NIAMDD staffers have appeared on these radio and TV shows.

Special Events:

The major news event at NIH during FY 1973 involved very heavy demands upon the NIAMDD information staff for well over a week. An NIAMDD intramural scientist, Dr. C. B. Anfinsen, was awarded the Nobel Prize in Chemistry for his research into the structure and function of the enzyme ribonuclease, and immediately telephone calls and requests for background information and photographs swamped the NIAMDD Information Office. Arrangements were made for press conferences and radio and TV interviews with Dr. Anfinsen.

In conjunction with the NIH News and Features Section, a special 28-page photo portfolio was issued to illustrate the work being performed by some of the scientists who are conducting NIAMDD-supported research on the problem of digestive diseases.

In connection with the NIH Lecture, the NIAMDD as the host Institute assumed the responsibility for publicizing the Lecture. This included the preparation of appropriate releases to the press, an article for the NIH Record, and various publicity material including test cards, posters, etc.

Publications and Reports:

Three separate NIAMDD publications either were undergoing revision or preparation during FY 1973. The Information Office's most recent effort involves the preparation of a pamphlet on Vitamin E, a vitamin in which the public interest has been stimulated in the last year or so. Undergoing revision are a fact sheet on hypoglycemia, which continues to arouse a great deal of interest, and a fact sheet concerning the use of bee venom for the treatment of rheumatoid arthritis and other forms of arthritis.

During the past fiscal year the Institute continued to give impetus to its Equal Employment Opportunity program. In this regard, an Information Office staff member wrote and edited six EEO ACTION Newsletters to inform employees about the latest events regarding the program.

Most of the regular and special program reports required of the Institute by the Congress, DHEW, and NIH are based upon the "weekly reports", brief summaries of both intramural and extramural research accomplishments. During Fiscal Year 1973 there were a number of significant findings in both basic research and in clinical research, with the emphasis on the latter.

Characteristic weekly reports during FY 1973 included the demonstration that the affected skin of psoriatic patients is deficient in cyclic AMP; correction of the metabolic defects in Fabry's disease via kidney transplantation; modification of an oral diet for intravenous use that permits survival of two-thirds of patients in acute renal failure; development of a method whereby porcine insulin can be converted easily and inexpensively to human insulin, and continued success in the dissolution of long-standing cholesterol gallstones via oral administration of a primary bile acid.

More Information Office staff time is allotted annually to preparation of exhaustive special reports and other material in connection with the Congressional appropriation hearings than is allotted to any other single project. During Fiscal Year 1973 special reports were prepared on arthritis, diabetes, digestive diseases, hematology, kidney disease, cystic fibrosis and nutrition, in addition to the Director's opening statement, the budget narrative, and highlights of research. A significant amount of time is also spent on collaborating with other Institute information staffers concerning the preparation of a number of reports which involve two or more Institutes.

Public Inquiries:

One of the most important functions of the Information Office is answering public inquiries which emanate from a wide variety of groups, ranging from people with illnesses, and school children with science fair projects, to Congressmen, scientific researchers and practicing physicians. A large amount of staff time is involved in handling such requests, often at considerable savings of the time of NIAMDD scientific and administrative personnel. Inquiries often may require specific and hard to obtain information that is not easily available.

During Fiscal Year 1973, the Information Office responded to 2,527 public inquiries, 322 Congressional inquiries, and 165 inquiries from representatives of the various news media. These requests for information or photographs are usually made either by letter or by telephone, and Information Office staffers usually respond in kind. Also, during FY 1973, the Information Office distributed 104,000 publications, necessitating overtime employment on weekends to handle the requests.

Exhibits:

The Information Office also conducts the NIAMDD program of scientific exhibits. This is a major source of professional education. NIAMDD displayed five different exhibits during FY 1973 at eight medical and scientific meetings throughout the country, which were viewed by approximately 65,000 physicians, scientists and paramedical people. Institute exhibits on cystic fibrosis, artificial kidney, human growth hormone, and gout were among those shown. Institute publications and reprints are distributed in conjunction with these exhibits. Information Office staff members, including the exhibit specialist, write press releases, reports, etc., as needed.

OFFICE OF THE DIRECTOR

Summary

Fiscal Year 1973

Dr. G. Donald Whedon, Director
Dr. Ronald W. Lamont-Havers, Deputy Director
Mr. F. L. Mills, Executive Officer

The Office of the Director has overall responsibility for directing and coordinating basic laboratory research; clinical investigations; extramural activities such as research grants, research fellowships, and training grants; contract research and developmental programs; and epidemiologic and clinical field studies. In addition, this Office cooperates with other scientific organizations in coordinating medical research in the Institute's fields of interest; cooperates and maintains liaison with other Institutes and constituents of other Federal agencies; and participates in determining policy governing the National Institutes of Health. It is also responsible for the collection and dissemination of informational material to interested professional and lay individuals and groups.

The continuing review of the Institute's organizational structure to better identify the areas of responsibility has resulted in the establishment of new branches and sections in the Intramural Research area of the Institute as well as the reorganization of the Extramural Program Area into five subdivisions, or "clusters". The reorganization of the Extramural Program Area and the changes involved are included in the report of the Extramural Program which precedes this summary.

On May 19, 1972, H.R. 13591 (P.L. 92-305) was signed changing the name of the Institute to the National Institute of Arthritis, Metabolism, and Digestive Diseases. The name reflects more adequately the Institute's interest in digestive diseases and renewed emphasis on this group of disorders. The Act changing the name also created a Committee to advise the Director of the Institute concerning digestive diseases. In addition a position of Associate Director for Digestive Diseases was established.

A new Laboratory of Chemical Physics was established consisting of three sections previously located in the Laboratory of Physical Biology. Two additional new sections were established in the Laboratory of Chemical Physics: (1) Section on Spectroscopy and Structure, and (2) Section on Membrane Biophysics. A new section on Protein Conformation was established in the Laboratory of Chemical Biology.

Several new appointments have been made to key positions within the Institute during this fiscal year. Dr. Ronald W. Lamont-Havers was appointed Deputy

Director of the Institute. Mr. F. L. Mills was appointed Executive Officer of the Institute; Dr. Lionel M. Bernstein was appointed Acting Associate Director for Digestive Diseases and Nutrition; Dr. Edward P. Offutt, Acting Associate Director for Extramural Program Activities. Dr. Martin Gellert was appointed as Acting Chief of Laboratory of Molecular Biology. Dr. Elizabeth Neufeld was appointed Chief of the Section on Human Biochemical Genetics, Arthritis and Rheumatism Branch. Dr. Hiroshi Taniuchi was appointed Chief, Section on Protein Conformation. The name of the Section on Diabetes and Intermediary Metabolism, Clinical Endocrinology Branch was changed to Section on Diabetes. A new section on Diseases of the Liver was established in the Metabolic Diseases Branch and Dr. Paul D. Berk was appointed Chief of the Section. Dr. Stephen E. Dippe was appointed Associate Chief, Southwestern Field Studies Section.

The activities of the Office of Public Information are included in a separate report which precedes this summary. Efforts continue to inform the general lay public of the Institute's mission and activities. Responses to 165 press inquiries and 2527 public inquiries were made during the year, in addition to responses to 322 congressional inquiries which were made by this office. Scientific exhibits were displaved at various medical and scientific meetings throughout the country which were viewed by approximately 65,000 physicians, scientists and paramedical people, and over 104,000 publications were distributed.

Under the decentralized contracting operation, relations with other organizational elements within and outside of the Institute and with the contracting community in general have continued to be good. The volume of activity continued at about the same level as in Fiscal Year 1972. Near the end of the Fiscal Year some decrease occurred in the Artificial Kidney Program. Hormone distribution, scientific communications, and augmentation of intramural research remained about the same. New contracts were awarded for: (1) development of drugs for the early diagnosis and treatment of "Hurler's Syndrome"; (2) an evaluation to determine research needs in the field of digestive diseases; and (3) an evaluation of the effectiveness of the National Pituitary Agency on the field of endocrinology. The multi-clinical center study of the treatment of Crohn's Disease continued into its second year. Another contract, which was mentioned in the 1972 report as being initiated, was not awarded. This was to have been a study to measure the impact of NIH research and training support on the quality of patient care service provided by institutions receiving such support. A contract was awarded to support a multi-clinical center trial on the dissolution of gallstones by bile acid feeding. This clinical trial is to continue under contract for a period of at least five years. The use of multiple-year contracts and incremental funding was relied upon at an increasing frequency during Fiscal Year 1973.

Management continues to place emphasis on mission-oriented training opportunities for all employees of the Institute. Part V of the Basic Ideas in Biology (microbiological laboratory skills and animal handling) has been completed by the Institute's Personnel Office. Because of the continued interest in this particular series of training, Part I of the Basic Ideas in Biology (basic mathematics and laboratory skills) was initiated again this year. An additional course, Basic Ideas in Chemistry (elementary techniques of chemistry, metric system, titration, chromatography, density determinations) was initiated primarily for employees who had completed all sections of the Basic Ideas in Biology and who were interested in continuing their training in the sciences. During the year, short-term training opportunities were arranged for 45 secretarial-clerical employees, 5 supervisory managerial personnel and 115 members of the professional staff.

Equal Employment Opportunity

Restrictions on employment and promotions as a result of ceiling adjustments and grade de-escalation requirements have curtailed the progress and accomplishments of many of the elements of the EEO program or documented in the NIAMDD Affirmative Action Plan. Despite these restrictions we have made considerable progress in some of these areas. For example, job restructuring at the lower grade levels; appointment of an EEO Coordinator, Mr. Joe Ager, who has devoted many months of full-time activity in implementation and strengthening the total EEO Program for this Institute; and increasing emphasis on training.

Emphasis continues toward making all employees more aware of the EEO Program and the need for their participation in making the NIAMDD Affirmative Action Plan a reality. This is being done through meetings of the individual Laboratories and Branch staff to stress this program. An EEO meeting of all Institute employees was held in the Jack Masur Auditorium in February. Regular meetings of black and women employees are continuing and the EEO Newsletter is published as items of interest are developed.

The NIAMDD EEO Advisory Committee, chaired by the Director, continues to play an active role in EEO matters. This group continues to represent all levels of employees within the Institute.

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